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THE PAPER CHROMATOGRAPHIC FRACTIONATION OF PLANT STERYL ESTERS[1]

A. KUKSIS AND J. M. R. BEVERIDGE

Abstract

The even-numbered C_2–C_{22} saturated and the C_{18}-unsaturated fatty acid esters of beta- and gamma-sitosterol, stigmasterol, ergosterol, stigmastanol, and gamma-sitostanol were used separately and in model mixtures for the evaluation of existing and the development of new paper chromatographic methods for the separation and identification of these compounds. Satisfactory separations of the esters were obtained by the ascending reversed phase chromatography on cellulose or glass fiber paper using one of the following systems depending upon the number of carbon atoms in the fatty acid moiety of the ester: C_2–C_{12}, aqueous acetic acid/chloroform/paraffin oil; C_8–C_{18}, aqueous propionic acid/paraffin oil; C_8–C_{22}, acidified aqueous methanol/chloroform/paraffin oil or aqueous butyric acid/paraffin oil. The rate of migration of the esters was inversely proportional to the molecular weight and was increased by the introduction of unsaturation.

The increased movement due to unsaturation has been correlated with the number of ethylenic hydrogens, and the possibility that polarity of these atoms might account for this phenomenon is discussed. The reversed phase paper partition chromatography of sterols as their esters is suggested as a potential tool for evaluation of polarity of the various attributes of the sterol nucleus in terms of the chain length of the ester fatty acid. The rule of independent contributions of the constituents of a molecule to its distribution isotherm was observed not to hold in this fatty ester series.

Introduction

In studies on the chemical composition of certain corn oil distillates, a method was required for the fractionation and identification of the various steryl esters present. Since no directly applicable method appeared to have been described, attempts were made to adapt the procedure of reversed phase partition chromatography on paper, a method of already proved value for the separation of certain cholesteryl esters (1, 2). A number of reversed phase partition systems were investigated. Those containing aqueous aliphatic alcohols or organic acids with or without added chloroform proved to be the most satisfactory. This report describes the chromatographic behavior of the C_2–C_{22}-saturated and the C_{18}-unsaturated fatty acid esters of beta- and gamma-sitosterol, stigmasterol, ergosterol, stigmastanol, and gamma-sitostanol in such systems.

The following systems are described in detail: aqueous acetic acid/chloroform/paraffin oil (modified from Michalec (2)), aqueous propionic acid/paraffin

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oil, aqueous butyric acid/paraffin oil, acidified aqueous methanol/chloroform/paraffin oil (modified from Labarrere *et al.* (1)).

Since the completion of these studies a paper (3) has appeared describing the column chromatographic fractionation of some cholesteryl esters on silicic acid.

Materials and Methods

Steryl Esters

These were prepared in this laboratory (4) from the respective acid chlorides and purified sterol samples. Detailed methods of preparation, physical constants, and certain other properties will be described elsewhere.

Reagents and Solvents

Antimony trichloride (Analar) and liquid paraffin (Mineral Oil Light White) were obtained from British Drug Houses (Canada) Limited. Dow Corning Silicone Fluid 200 and 550 were obtained from Dow Corning Silicones Limited, 1 Tippet Road, Downsview, Toronto, Ontario. Other reagents and solvents were ordinary laboratory chemicals of reagent grade and were used without further purification.

Final Elution Systems

Mobile phase.—System A, methanol/chloroform/water/acetic acid, 50/50/5/5 by volume, equilibrated with paraffin oil; system B, propionic acid/water, 90/10 by volume, equilibrated with paraffin oil; system C, acetic acid/chloroform/water, 75/20/5 by volume, equilibrated with paraffin oil; system D, butyric acid/water, 75/25 by volume, equilibrated with paraffin oil. In all cases the equilibration with paraffin oil was accomplished by shaking the aqueous mobile phases with about 5% of their volume of paraffin oil for 30 minutes and then allowing to stand till clear (48 hours).

Stationary phase.—Paraffin oil supported on paper served as the stationary phase for all systems.

Impregnation of Papers

The paraffin oil to be used for impregnation was first saturated by being shaken for 30 minutes with about 5% of its volume of the aqueous alcohol or acid used in the mobile phase. After the phases were allowed to separate, 50 ml of the clear paraffin phase was diluted to 1000 ml with chloroform and transferred to a wide-mouth container. Strips of Whatman No. 1 and No. 3 MM filter paper (14×45 cm) and Reeve Angel No. X-934-AH glass fiber paper (10×37.5 cm) were drawn through the solution, blotted uniformly between sheets of filter paper, and dried by hanging in air at room temperature. After 1 hour most of the solvent had evaporated and the strips were ready for chromatography. They could be kept overnight without any detrimental effect.

Application of Steryl Esters to the Paper

For chromatography the impregnated paper strips were cut into standard lengths of 7×45 cm and 2×37.5 cm for the cellulose and glass fiber paper respectively. Individual steryl esters or mixtures were applied (50 μg each ester per square centimeter) in 1% chloroform solution as a band (0.5 cm wide) from edge to edge of the paper about 5 cm from the lower end of the strip.

Method of Chromatography

The chromatography was carried out at room temperature (approximately $25 \pm 1°$ C), by the ascending technique, in glass jars 15 cm in diameter and 45 cm high containing 100 ml of the mobile phase on the bottom. The paraffin-impregnated paper strips were suspended above the solvent by means of Teflon clips from glass rods bent to fit the jars tightly from inside. The jars were closed with glass plates sealed in place with modelling clay, and equilibration was allowed to proceed for 4 hours. At the end of this time the strips were lowered into the mobile phase with the minimum of disturbance of the equilibrium attained and the run began. Satisfactory separations could usually be effected without prior equilibration. After the solvent front had ascended about 35 cm (from 12 to 48 hours depending on the system) its location was marked and the papers dried by hanging in a stream of air.

Location of Steryl Esters

For identification the dried chromatograms were cut into strips (0.5 cm wide), one of which was stained with iodine vapor for unsaturation, and another with antimony trichloride (25% in glacial acetic acid) for sterols. Others, if necessary, were sprayed with 27 *N* sulphuric acid or a 1:10 mixture of concentrated sulphuric acid:acetic anhydride and gently heated. The latter reagents permitted a more satisfactory location of the free and esterified saturated sterols, particularly on glass fiber filter paper. The various strips were then compared and the locations of the esters and their identity decided. The unsaturated sterols and their esters developed pink colors readily when the paper strips were dipped into the antimony trichloride solution, blotted between sheets of filter paper, and warmed on a white enamel plate on a steam bath. The saturated sterols and their esters, however, required about a day at room temperature following the short warming period on the steam bath for color to develop.

Screening of Potential Solvent Systems

The evaluation of potential solvent systems for the chromatographic fractionation of the steryl esters consisted of performing systematic test runs with various mobile phases against a chosen stationary phase (paraffin or silicone oil) under the experimental conditions described above. A mixture of stigmasterol and its acetic, lauric, and stearic acid esters in approximately equal amounts was used as a test sample. To 95 ml of the aliphatic alcohol or acid under consideration was added 5 ml of water and, after equilibration of this solution with paraffin or silicone oil, its effect on the movement of the sterol and its esters was determined. Absolute alcohols or acids could not be used as they, even after careful equilibration with the oil, were observed to wash out the stationary phase. If the ester moved with the solvent front or close to it, indicating that the mobile phase was not sufficiently polar,* the water content was increased 5 ml at a time till the R_f values were depressed to the desired level. After each addition of water the phases were allowed to equilibrate and any oil forced out of solution was removed. When the esters stayed at the origin indicating that

*The term "polar" or "non-polar" is used throughout the paper in the same sense as defined by Cassidy (5).

the solvent was too polar, chloroform was added 5 ml at a time till the R_f values were increased to the desired level. After each addition of chloroform more paraffin oil was added, the phases equilibrated, and any extra oil removed. Promising systems yielding desirable R_f values, compact bands, and good reproducibility were adjusted further with smaller increments of water or chloroform as required, and tested with a wider selection of steryl esters. Those systems that did not show these characteristics under the preliminary conditions were not further examined.

Results and Discussion

Solvent Sytems

From an examination of a large number of solvent systems suggested for lipid chromatography by Schlenk *et al.* (6), Labarrere, Chipault, and Lundberg (1) concluded that only mixtures of chloroform, methanol, and water appeared promising. However, they noticed that the addition of acid to their systems considerably improved the resolution and indeed Michalec (2) and Zimmerman and Brase (7) had already shown that aqueous acetic acid and chloroform mixtures were suitable for the separation of at least the shorter-chain fatty acid esters of cholesterol. From these reports it seemed quite possible that the chloroform in the aqueous methanol or acetic acid solutions served only to lower the polarity of the mobile phase. If this were the case then the use of aqueous solutions of aliphatic acids higher than acetic or of alcohols higher than ethanol would provide simpler and more stable media of appropriate polarity without the use of chloroform. In respect of the aliphatic acids this possibility was realized but due to the relative immiscibility of the higher alcohols with water the latter class of compounds did not lend themselves to the development of such media.

Of the aliphatic alcohols examined (methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-amyl) none appeared to give aqueous mixtures which could be directly used for the fractionation of steryl esters. While the aqueous mixtures of methyl, ethyl, and propyl alcohols were too polar, those of the other alcohols were not polar enough, and their polarity could not be increased by the addition of more water since the solubility limit had been reached.

The addition of chloroform to aqueous methyl or ethyl alcohol, however, produced differential migration of the steryl esters readily, and an approximately 1:1 mixture of 95% alcohol and chloroform gave fair spacing of the various ester bands. The addition of a few milliliters of acid depressed the R_f values somewhat but sharpened the resolution. The formic acid used by Labarrere *et al.* (1) for acidification of similar systems could be replaced by acetic or dilute hydrochloric acid. The system deteriorated slowly probably due to the volatility of its components and possible ester formation. A methanol/chloroform/water/acetic acid ratio of 50/50/5/5 by volume, equilibrated with paraffin oil (system A), was found to be satisfactory for the separation of the C_8–C_{22} fatty acid esters of all the sterols examined except the more polar ergosteryl esters. Though the other alcohols were not examined further, it is likely that they also can be adapted to the formation of acceptable

partition systems when diluted with suitable miscible polar solvents, e.g. cellosolves.

Aqueous solutions of formic, acetic, propionic, and butyric acids were investigated but only the propionic and butyric solutions could be utilized directly. Propionic acid/water in a volume ratio of 90/10, equilibrated with paraffin oil (system B), was particularly effective in the separation of the C_8–C_{18} fatty acid esters. Butyric acid/water in a volume ratio of 75/25, equilibrated with paraffin oil (system D), gave R_f values very similar to those obtained with system A. The unpleasant odor of this solvent system, however, was considered a disadvantage. The formic and acetic acids were too polar and their simple aqueous solutions effected no movement of the esters at all. On dilution with a non-polar solvent such as chloroform or acetone, systems could be devised which produced distinct ester bands with satisfactorily spaced R_f values. Acetic acid/chloroform/water in a ratio of 75/20/5 by volume, equilibrated with paraffin oil (system C), gave a fair resolution of the steryl esters of C_2–C_{12} fatty acids and the ergosteryl esters of long-chain fatty acids.

Attempts to utilize the alcohol/acetone mixtures with and without formic acid as described by Labarrere *et al.* (1) for the separation of unsaturated esters from one another, and from the group of saturated esters, failed since the latter, contrary to expectations, moved.

The aqueous organic acid systems, particularly those of propionic and butyric, were the most reproducible and gave the best resolution. With reasonable care, however, the other systems also performed quite satisfactorily, as indicated by the R_f values collected in Table I.

For optimum resolution of the ergosteryl esters, the mobile phases should be slightly more polar than those necessary for the separation of the corresponding esters of the more saturated sterols.

Stationary Phases and Supporting Media

The solvent compositions for optimum resolution were found to be fairly critical for a given set of experimental conditions (nature and amount of stationary phase, paper, temperature, etc.) and minor modifications in these occasionally required considerable readjustment in the solvent ratios. In doing this, however, little difficulty was experienced when the system outlined above for screening of potential solvent systems was followed. The two stationary phases investigated (paraffin and silicone oil) gave about the same ester separations.

Silicone oils have been reported to be less readily washed out of the paper than other stationary phases hitherto used, such as, for example, paraffin oil (8). However, in the systems investigated here and when proper equilibration with the mobile phase was achieved, the paraffin oil mixture caused no difficulty. Furthermore, the latter offered a distinct advantage by permitting its adsorption chromatographic removal from the steryl esters after elution from the chromatograms in preparative paper chromatography.

The amount of the stationary phase retained by the supporting medium, when applied as above, depended upon the nature of the medium used and the concentration of the dipping solution. The utilization of a 5% (v/v) solution

TABLE I

R_f values* of various plant sterols and their esters in different reversed phase partition systems†

	Compounds																	
	Ergosterol			Stigmasterol			γ-Sitosterol			β-Sitosterol			γ-Sitostanol			Stigmastanol		
System:	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
Free sterol	1.00	1.00	0.90	1.00	0.97	0.90	0.98	0.91	0.85	0.97	0.90	0.80	0.91	0.88	0.79	0.90	0.88	0.80
Acetate	1.00	0.91	0.67	1.00	0.85	0.63	0.98	0.82	0.62	0.97	0.80	0.56	0.95	0.79	0.58	0.93	0.80	0.52
Butyrate	1.00	0.87	0.62	0.96	0.79	0.55	0.95	0.77	0.50	0.92	0.73	0.45	0.92	0.74	0.46	0.90	0.72	0.41
Caproate	0.96	0.80	0.53	0.91	0.73	0.43	0.90	0.67	0.38	0.84	0.64	0.36	0.85	0.63	0.35	0.83	0.61	0.32
Caprylate	0.90	0.73	0.42	0.83	0.62	0.35	0.78	0.52	0.33	0.74	0.50	0.30	0.73	0.49	0.28	0.71	0.50	0.25
Caprate	0.83	0.64	0.33	0.72	0.49	0.29	0.67	0.45	0.25	0.64	0.39	0.22	0.62	0.39	0.21	0.60	0.38	0.20
Laurate	0.73	0.50	0.27	0.62	0.38	0.22	0.57	0.35	0.18	0.54	0.32	0.16	0.52	0.32	0.16	0.50	0.30	0.15
Myristate	0.64	0.40	0.21	0.52	0.31	0.16	0.47	0.28	0.15	0.44	0.26	0.13	0.43	0.25	0.12	0.41	0.24	0.12
Palmitate	0.55	0.30	0.16	0.42	0.24	0.14	0.39	0.23	0.13	0.37	0.20	0.12	0.34	0.20	0.12	0.32	0.20	0.11
Stearate	0.45	0.25	0.13	0.35	0.21	0.12	0.33	0.18	0.11	0.30	0.16	0.11	0.29	0.17	0.11	0.27	0.16	0.10
Arachidate	0.36	0.20	0.13	0.28	0.16	0.10	0.27	0.15	0.11	0.24	0.13	0.10	0.22	0.13	0.11	0.20	0.13	0.10
Behenate	0.30	0.15	0.13	0.22	0.13	0.10	0.20	0.12	0.11	0.19	0.11	0.10	0.17	0.11	0.10	0.15	0.10	0.10
Oleate	0.56	0.28	0.16	0.40	0.26	0.15	0.39	0.20	0.12	0.36	0.18	0.12	0.32	0.22	0.11	0.21	0.21	0.10
Linoleate	0.62	0.38	0.20	0.50	0.29	0.16	0.45	0.27	0.15	0.43	0.23	0.13	0.40	0.27	0.12	0.42	0.24	0.11
Linolenate	0.70	0.52	0.25	0.59	0.34	0.21	0.53	0.33	0.18	0.51	0.30	0.15	0.48	0.30	0.16	0.48	0.30	0.15

*Averages of at least three determinations differing by less than 0.01 R_f unit.

†Mobile phase:

System A: Methanol/chloroform/water/acetic acid, 50/50/5/5 by volume, equilibrated with paraffin oil.

System B: Propionic acid/water, 90/10 by volume, equilibrated with paraffin oil.

System C: Acetic acid/chloroform/water, 75/20/5 by volume, equilibrated with paraffin oil.

Stationary phase:

Paraffin oil supported on paper served as stationary phase for all systems.

of the silicone or paraffin oil in chloroform for the impregnation permitted the preparation of satisfactory chromatograms with all the papers tested. The actual amount of the oil retained by any particular type of paper varied and so did the solvent ratios necessary for the optimum resolution of the esters. An increase in the amount of the stationary phase permitted the handling of larger amounts of ester but also slowed the rate of migration. The use of the glass fiber paper in these separations offered little advantage over the cellulose fiber papers, except that the former provided a means for a more satisfactory location of the saturated fatty acid esters of the saturated sterols.

Interferences

Triglyceride esters and fatty acid esters of long-chain alcohols move approximately the same distance as the steryl esters, whereas mono- and di-glycerides, free fatty acids and their simple aliphatic esters, and free sterols migrate further and can be separated from most of the steryl esters. Although these substances, with the exception of the free sterols, do not interfere with the color tests used for identification, their presence exercises a marked influence on the R_f values of the steryl esters. When fast-moving interfering substances, in amounts similar to or greater than the steryl esters, are present, the R_f values of the esters are increased, whereas the presence of relatively large amounts of slow-moving contaminants has the opposite effect. If possible, these contaminants should be removed in advance by some means such as adsorption chromatography, molecular distillation, etc. Similarly, the esters themselves also interfered with the movement of each other, and the R_f values observed were either higher or lower than those recorded for the individual steryl esters depending on whether the faster- or the slower-moving steryl esters predominated in the mixture under investigation. This phenomenon, however, does not constitute a serious drawback to the application of this procedure to the examination of natural steryl ester mixtures since these do not ordinarily contain significant amounts of the fast-(acetates, butyrates) or slow-(behenates, arachidates) moving esters.

Behavior of Steryl Esters in Reversed Phase Systems

Table I lists the R_f values obtained for the free and esterified sterols using the partition systems A, B, and C. These values were recorded by chromatographing the esters individually and represent averages of at least three separate determinations, which differed by no more than 0.01 R_f unit. It can be seen that the esters were separated primarily on the basis of their molecular weights. The lower molecular weight derivatives (acetates, butyrates, caproates; R_f values of the order of 0.90 to 0.60) of any particular sterol moved faster than those of higher molecular weight (stearates, arachidates, behenates; R_f values of the order of 0.20 to 0.10). These results confirm the observation (1) that with selection of suitable solvent systems a difference in chain length of two methylene units is sufficient for a successful chromatographic separation of even the long-chain fatty acid esters of sterols. With the exception of ergosterol and stigmasterol, differences in the sterol moieties of these esters appear to exercise only a minor effect on the migration. Thus the same fatty acid esters

of beta- and gamma-sitosterol and of their saturated sterol analogues move at approximately the same rates in all systems.

The incorporation of ethylenic linkages in the sterol or the fatty acid part of the ester increased the R_f beyond the amount expected from the negligible change in molecular weight, but the degree of increase was dependent on the location of the unsaturation. The introduction of one, two, and three double bonds into the fatty acid moiety of the ester, as in oleates, linoleates, and linolenates, showed an effect approximately equal in terms of R_f values to shortening the fatty acid chain length by two, four, and six methylene units, respectively. As a result the oleic acid ester of a particular sterol overlapped with that of palmitic acid, while the linoleic and linolenic acid esters superimposed to a considerable extent on the myristic and lauric acid esters of the corresponding sterol, respectively.

The introduction of a double bond into the sterol side chain (cf. beta-sitosterol and stigmasterol, Fig. 1) appeared to affect the movement of the

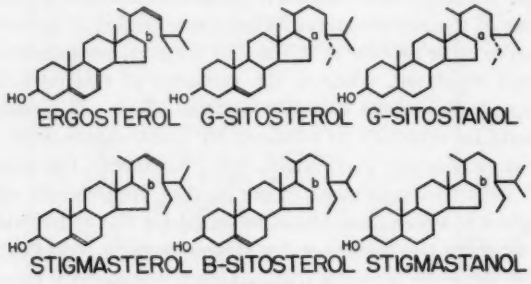
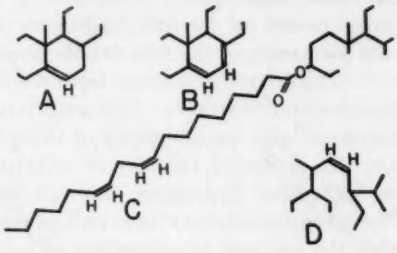


FIG. 1

corresponding steryl esters very much like the introduction of a double bond into the fatty acid chain and was equal in terms of R_f values to shortening the latter by two methylene units. This resulted in the superposition of beta-sitosteryl esters with the corresponding next higher even-numbered stigmasteryl esters.

The introduction of unsaturation into the sterol nucleus, on the other hand, did not appear to have a comparable effect. Thus, for example, the beta-sitosteryl palmitate could not be effectively separated from stigmastanyl palmitate. Only after the introduction of two nuclear double bonds (ergosterol, Fig. 1) was there produced an effect approaching that observed with non-nuclear double bonds. Thus, an examination of the behavior of the ergosteryl esters in these systems reveals that the R_f values agree closely with those expected for a steryl ester with two nuclear double bonds, when calculated utilizing the corresponding stigmasteryl esters as the reference compounds and allowing one methylene unit for the difference in molecular weight. From these observations it can be estimated that the effect of the introduction of one nuclear double bond of the kind present in these sterols is equal in R_f value to shortening the fatty acid chain by one methylene unit.

The relatively small contributions of the individual nuclear double bonds to the rate of migration of the steryl esters, in comparison to the effect of side chain and fatty acid residue unsaturation, might be explained if the apparent double bond polarity could be attributed to the total number of ethylenic hydrogens present. Thus, the partial structures A–D illustrate that the double bonds in the fatty acid residues (C) and the sterol side chains (D) furnish two hydrogens, whereas each nuclear double bond in these sterols (A and B)



supply only one double-bond hydrogen each. On this basis the introduction of one ethylenic hydrogen would be equivalent in R_f values to shortening the fatty acid ester chain by one methylene unit. Whether this relationship is incidental or manifests a true physicochemical property cannot be definitely stated from the limited number of cases examined here. The R_f values observed with these compounds in all of the above solvent systems, however, have repeatedly approximated this relationship, and appear to reflect the experiences of other workers with the steryl esters (1), free sterols (9), and fatty acids (10, 6) in similar reversed phase partition systems.

An examination of the literature for comparable phenomena brought up the case of the relatively acidic (polar) nature of acetylene and its monosubstitution products. It has been deduced on theoretical grounds that when an atom is joined to another by a multiple bond, it may be expected to hold its remaining electrons more firmly than in the case of a single bond (11). Thus, as far as the electrons taking part in a carbon–hydrogen bond in acetylene are concerned, the carbon is acting like an atom of a more electronegative element, such as nitrogen. Consequently, acetylene is more acidic than ethane, just as ammonia is more acidic than methane. A similar, but smaller, effect has also been suggested to operate in ethylenic compounds, so that a hydrogen joined to a doubly bonded carbon atom might show an intermediate acidity (11). This supposition is supported by the observation that benzene is a stronger acid than a paraffin hydrocarbon, but a much weaker one than acetylene (12). Although an acetylenic hydrocarbon is ordinarily considered to be acidic only if it has at least one hydrogen that is directly linked to a triply bonded carbon atom, it has been shown that 7,9-pentadecadiyne, with no directly linked hydrogens, is slightly acidic (13). Though this phenomenon has been rationalized as possibly arising from resonance stabilization of certain contributory structures, it might be taken to indicate that part of the electrical dissymmetry, residing in the triply bonded carbon atoms, may be passed on to the adjacent

carbons rendering their hydrogens more polar. In this connection it is interesting to point out that the introduction of an acetylenic linkage into the fatty acid molecule as in octadec-6-yenoic and octadec-9-yenoic acids increases the R_f value in comparison to the parent compound, stearic acid, to approximately the same extent as the introduction of two ethylenic linkages as in linoleic acid or the removal of four methylene units as in myristic (10). This observation could again be explained if it was postulated that the four hydrogens linked to the carbons adjacent to the triple bond ($—CH_2—C\equiv C—CH_2—$) had become polar to about the same extent as the four hydrogens attached to the four carbons involved in the formation of the two double bonds.

Additional support for this hypothesis arises from the arithmetic additivity of the individual polarizing contributions. This additivity is observed in the case of ethylenic, acetylenic, and combinations of these linkages (10, 14, 6).

The possibility that the increased polarity of unsaturated compounds is associated with these polarized hydrogens and not necessarily with any geometrical changes these compounds may have undergone is further supported by the observation that the *cis*- and *trans*-isomers of long-chain unsaturated fatty acids show approximately the same polarity (10, 14, 6) and are separated from each other only with great difficulty (15). The relative unimportance of configurational factors in the observed increased polarity of the unsaturated compounds is also indicated by the lack of difference in R_f values noted between compounds with multiple isolated double bonds and multiple conjugated double bonds (10).

The consistently observed slightly higher R_f values for the gamma-sitosteryl and gamma-sitostanyl esters in comparison to the corresponding beta-sitosteryl and stigmastanyl esters present a special case, and may be a result of the known differences in the stereochemical configurations of their sterol moieties (16). Similar observations with the free sterols (17, 18), however, have caused speculation (17) that gamma-sitosterol might not be the 24-alpha-ethyl-cholesten-5-en-3-beta-ol as now believed (16), but possibly a C_{28} compound.

On the basis of the experiments reported here and an examination of the literature, it is suggested that the ethylenic hydrogens are polar and that the rate of migration of the compounds studied is largely determined by the number of these hydrogens in the molecule. Further experimentation with steroids possessing nuclear double bonds with two or no ethylenic hydrogens in similar partition systems would provide a further test for the double-bond hydrogen polarity. The reversed phase chromatography of the long-chain fatty acid esters of such compounds should again prove to be an excellent tool for the evaluation of these localized electrical dissymmetry effects in terms of the length of the fatty acid chain.

Finally, these studies with several homologous series of fatty acid esters offered another chance for checking the rule of independent contributions of the different constituents of a molecule to its distribution isotherm (19). It has been shown that for those series where these contributions are truly independent, a straight line is obtained when the R_M values (20), which are equal to $\log 1 - R_f/R_f$, are plotted against the number of the carbon atoms in the fatty

acid chain. Since the fatty acids as their ammonium salts are known not to obey this rule (21), whereas a number of other derivatives do (22), it was thought to be of interest to analyze the behavior of the steryl esters of the fatty acids. Figure 2 shows such a plot for the members of the various homol-

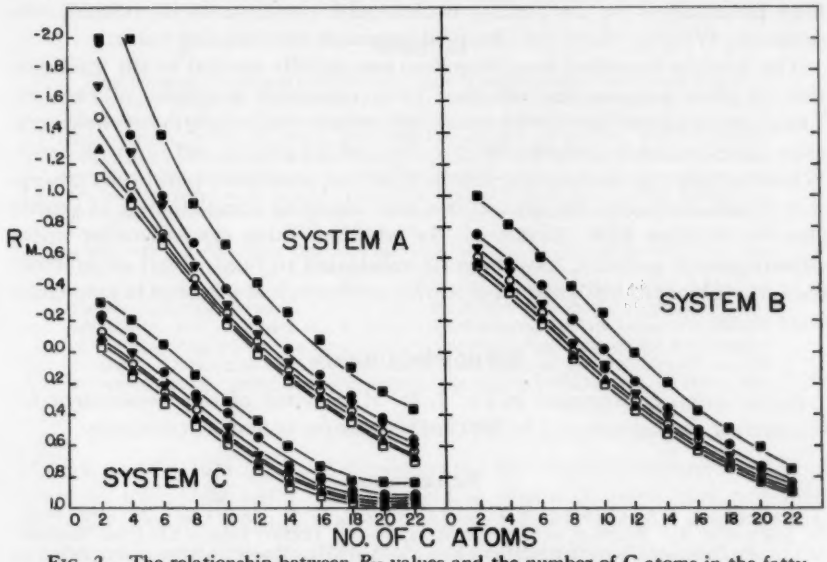


FIG. 2. The relationship between R_M values and the number of C atoms in the fatty acid chain for various steryl esters in solvent systems A, B, and C.
-■-■- ergosteryl esters, -○-○- beta-sitosteryl esters,
-●-●- stigmasteryl esters, -▲-▲- gamma-sitostanyl esters,
-▼-▼- gamma-sitosteryl esters, -□-□- stigmastanyl esters.
Systems A, B, and C as described in the text.

ogous series of steryl esters in three different solvent systems. Each curve represents a particular steryl ester, and the same characters on different curves refer to the same esters in the solvent systems A, B, and C. It may be noted that the straight-line relationship, if any, is obtained only over a part of the series, and may actually represent a section of a sigmoid curve describing the over-all behavior of the whole series in a given solvent system. It is pertinent to point out that those investigations (22) in which a straight-line relationship was reported dealt only with the short-chain fatty acid derivatives which would be expected on the basis of the work reported here to yield data approximating a straight line. The rest of the series shows a marked deviation from the expected linear relationship and approaches a limiting value as the number of carbon atoms increases. This effect is similar to that observed by Isherwood and Hanes (21), with the ammonium salts of the fatty acids, and resembles the observations of Michalec on the cholesteryl esters of formic, acetic, butyric, caproic, palmitic, and stearic acids (2).

The non-linearity of the migration rates might be explained if it was postulated that the introduction of a non-polar substituent (such as a methylene

unit) decreases the polarity of a strongly polar compound (such as an acetate) to a relatively greater extent than the polarity of a less polar compound (such as a stearate). This would render the higher members of the series somewhat more polar than they would have been, if the decrease in polarity had been proportional to the number of non-polar constituents introduced, consequently bringing about the observed approach to a limiting value.

The systems described here have been successfully applied to the fractionation of plant steryl esters obtained from molecular distillates of corn oil. These systems have also proved equally effective in the separation of cholesteryl ester mixtures, such as those obtained from blood plasma and egg yolk lipids. When working with natural steryl ester mixtures, previously purified by adsorption chromatography, the separations were about as complete and as readily effected as those from standards. In addition, these systems, after minor adjustments in polarity, have been demonstrated to bring about satisfactory resolutions of mono-, di-, and tri-glyceride mixtures, and mixtures of long-chain fatty acids.

Acknowledgments

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THE CALORIGENIC RESPONSE OF COLD-ACCLIMATED WHITE RATS TO INFUSED NORADRENALINE[1]

FLORENT DEPOCAS

With the assistance of VIATEUR SECOURS

Abstract

The increase in oxygen consumption during continuous intravenous injection of various doses of L-noradrenaline was measured in anesthetized rats fully acclimated to 6° C. The metabolic response was found to be linearly related to the logarithm of the amount of noradrenaline infused per minute. The calorigenic response to infusion of noradrenaline at a level of 1 μg per minute was then measured in rats undergoing acclimation to cold. The calorigenic response was found to increase with time of exposure to the cold environment, thus paralleling previous observations in the time course of gain in cold resistance, increase in food consumption, and loss of dependence on shivering during acclimation of white rats to cold. Also, the same maximal increase in oxygen consumption was obtained on infusion of noradrenaline into functionally eviscerated and sham-operated cold-acclimated rats, thus indicating that the liver and other abdominal viscera (kidneys excluded) are not involved in the calorigenic response to noradrenaline. It is proposed, as a working hypothesis, that striated muscle is the site of the major alteration in sensitivity to noradrenaline induced by acclimation to cold. Difficulties associated with consideration of striated muscle as the source of non-shivering thermogenesis in the cold-acclimated rat are discussed.

Introduction

Hsieh and Carlson (1) have demonstrated the marked calorigenic effect of intramuscularly injected L-noradrenaline in cold-acclimated rats and have suggested that this substance may be the mediator of the non-shivering heat production now well demonstrated (2, 3, 4) in the cold-acclimated white rat. In the present work, the intense calorigenic effect of noradrenaline was confirmed. Further observations were made on the dependence of the calorigenic response to the dose of intravenously infused noradrenaline and on the relationship between the calorigenic effect of an adequate level of continuously infused noradrenaline and the length of time of exposure of white rats to a cold environment. The effect of functional evisceration on the metabolic response to noradrenaline infusion in the fully cold-acclimated rat was also measured.

Methods

Calorigenic Response as a Function of Dose of L-*Noradrenaline*

Thirty-three male Sprague–Dawley rats weighing on the average 221 g (range 196 to 242 g) were randomly assigned to two groups maintained for 7 to 12 weeks at 30° C (4 rats) and 6° C (29 rats). They were kept in separate cages and Master Fox Chow and tap water were supplied ad libitum. After acclimation, when the warm-acclimated rats weighed 414 g (range 391 to 448) and the cold-acclimated rats weighed 374 g (range 304 to 416), the animals were anesthetized with sodium barbital (290 mg per kg, intraperitoneal). One

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hour after anesthesia they were taped to a board in the supine position in a room at 20° C, the trachea was cannulated, and the right femoral vein was exposed.

Oxygen consumption was measured with a Pauling O_2 analyzer and a dry test meter (American Meter Co.). The system had a lag of approximately 3 minutes. The P_{O_2} and volume of CO_2-free outlet gases were recorded, and the appropriate equation corresponding to these conditions (5) was used to calculate the O_2 consumed per unit time.

After 30 minutes, L-noradrenaline infusion into the femoral vein was started. The solution was delivered from a 2-ml hypodermic syringe mounted on a variable speed infusion pump (Harvard Apparatus Co. Inc. Model 600–900) set to deliver 0.00803 ml solution per minute. L-Noradrenaline bitartrate 0.2% (Levophed, Winthrop Laboratories of Canada Ltd.) was diluted with either 5% glucose solution or 0.1% sodium bisulphite to give the required concentration of amine.

In all experiments, oxygen consumption was then measured for 100 minutes after the start of infusion, readings being taken at intervals of 5 minutes. Due to variations in the response pattern observed in different animals the calorigenic effect of noradrenaline was estimated in the following way. The oxygen consumption values of each rat (1 ml O_2 = 1 cm) were plotted against time (5 minutes = 1 cm) on metric paper and the area under the curve during noradrenaline infusion was measured with a planimeter (Fig. 1). In these units a metabolic response of 100 cm^2 would correspond to an average increase of 5 ml O_2 per minute during a period of 100 minutes. The value of the calorigenic response was obtained by subtracting from the area corresponding to noradrenaline infusion the area corresponding to the average initial oxygen consumption measurements for the same period of time. Colonic temperatures were measured throughout with a copper–constantan thermocouple inserted to a depth of 6 cm.

Calorigenic Response during Acclimation to Cold

Thirty-four male Sprague–Dawley rats weighing on the average 210 g (range 166 to 241) were transferred from room temperature to a room at 30° C for 14 to 18 days. From this group, 22 rats were transferred to a room at 6° C and at appropriate intervals of time, the calorigenic response to a dose of 1 μg noradrenaline per minute, infused at a constant rate for 100 minutes, was determined as above. The same measurements were also made on 11 rats left at 30° C. For this series of measurements, measurements of initial oxygen consumption were made in a room at 30° C and the temperature of the room was then lowered to 20° C about 10 minutes after start of noradrenaline infusion. The oxygen consumption of three warm- and three cold-acclimated rats exposed to the same environmental conditions but without noradrenaline infusion was also measured. Colonic temperatures were measured throughout with a copper–constantan thermocouple inserted to a depth of 6 cm. Tests were performed at random during morning or afternoon.

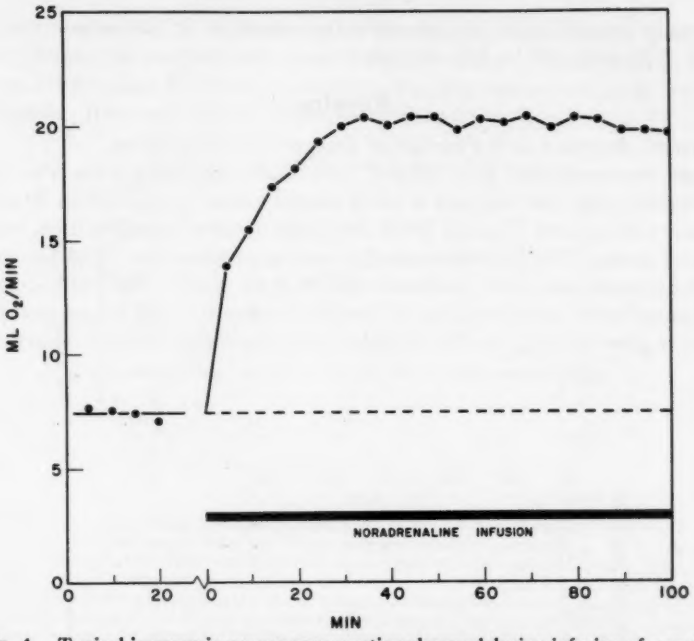

FIG. 1. Typical increase in oxygen consumption observed during infusion of noradrenaline into an anesthetized cold-acclimated rat at 200° C. The animal weighed 369 g, had been at 6° C for 61 days, and was given 1.48 μg noradrenaline base per minute, intravenously, from 0 to 100 minutes. The area between the oxygen consumption curve and the broken line (corresponding to average initial oxygen consumption) was taken as the metabolic response.

Calorigenic Response in Functionally Eviscerated Cold-acclimated Rats

In these tests, nine 6° C acclimated rats and two 30° C acclimated rats were used. Before transfer to the acclimation rooms the average weight of all the rats was 206 g with a range of 190 to 230 g. When used, the cold-acclimated rats (three rats per group) weighed on the average 359 g (range 326–395 g) and the two warm-acclimated rats weighed respectively 372 and 437 g. The animals had been acclimated to their respective environment for an average period of 61 days (range 52–70 days).

The animals were anesthetized and initially prepared as described above (in the calorigenic response vs. dose section), all experiments being conducted in a room at 30° C. Oxygen consumption was also measured in the same manner. Twenty minutes after start of oxygen consumption measurements the abdominal cavity was opened and threads were passed under the rectal colon, the coeliac and superior mesenteric arteries, the portal vein, and the oesophagus (6). Twenty minutes later the animals were either (*a*) eviscerated by tightening the threads already surrounding the blood vessels and 25 minutes later infused with 0.1% bisulphite in saline (6° C, EV.) for 100 minutes or (*b*) eviscerated and later infused with the same solution containing in addition noradrenaline at a concentration giving an infusion rate of 1 μg per minute (6° C, EV. + NA) or

(*c*) simply infused with the noradrenaline solution at the same rate (6° C, S.O. + NA) and (30° C, S.O. + NA).

Results

Calorigenic Response as a Function of Dose of L-*Noradrenaline*

When noradrenaline was infused into cold-acclimated rats, the oxygen consumption rose and reached a fairly steady value in from 20 to 30 minutes after start of infusion (Fig. 1). With the larger doses of noradrenaline, maximal values of oxygen consumption recorded were approximately 22 ml per minute. The integrated metabolic response observed in warm- and cold-acclimated rats infused with noradrenaline at levels between 0.1 and 3.0 μg per minute per rat is given in Fig. 2. The metabolic response was linearly related to the

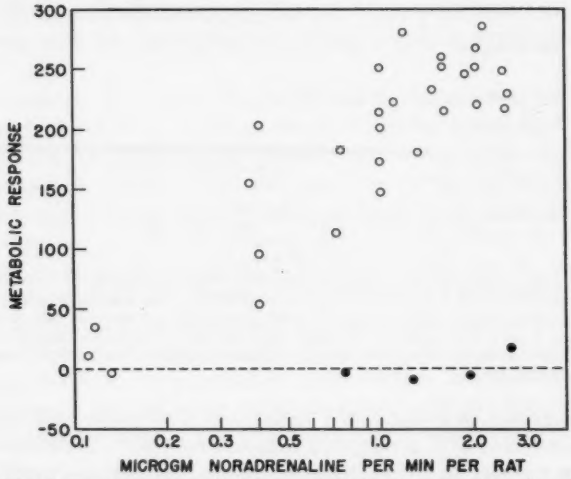

FIG. 2. Metabolic response to intravenously infused L-noradrenaline bitartrate in 30° C acclimated (●) and in 6° C acclimated rats (○). The metabolic response is given in square centimeters and corresponds to the area under the curve of oxygen consumption vs. time during noradrenaline infusion (100 minutes) minus the area corresponding to the initial oxygen consumption in the same period of time. The average increase in milliliter O_2 consumed per minute for each rat, during infusion of noradrenaline, can be obtained by dividing the metabolic response units by 20. The dose is in micrograms free base per minute per rat.

logarithm of the noradrenaline dose in cold-acclimated rats while the warm-acclimated rats gave little response within the same dose range. The average initial colonic temperature of all rats was 32.7° C (range 30.4 to 34.0) and changes of −1.9 to +9.8° C were observed after 100 minutes of noradrenaline infusion into 6° C acclimated rats, the larger increases being seen at the higher levels of infusion. The final average rectal temperature in the four 30° C acclimated rats was 28.9° C.

Calorigenic Response during Acclimation to Cold

The integrated metabolic responses observed during noradrenaline infusion into warm-acclimated rats and in rats exposed to cold for various lengths of

time are given in Fig. 3. In contrast to the warm-acclimated rats used in the dose–response experiments which showed no sensitivity to noradrenaline, all the warm-acclimated rats in this experiment gave a clear response to noradrenaline. The response, however, tended to decline with time of exposure to 30° C.

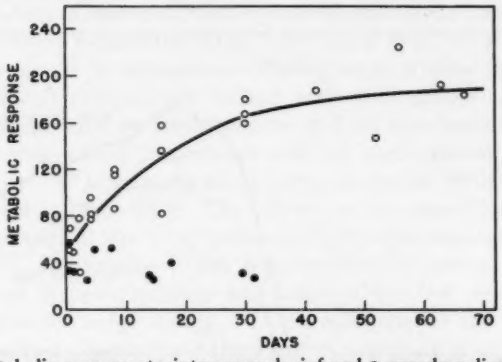

FIG. 3. Metabolic response to intravenously infused L-noradrenaline bitartrate at a level of 1 μg free base per minute per rat in rats previously maintained at 30° C (●) and 6° C (○). The metabolic response is given in square centimeters and corresponds to the area under the curve of oxygen consumption vs. time during noradrenaline infusion (100 minutes) minus the area corresponding to the initial oxygen consumption in the same period of time. The average increase in milliliter O_2 consumed per minute for each rat, during infusion of noradrenaline, can be obtained by dividing the metabolic response units by 20. All rats had spent 14 to 18 days at 30° C before time 0. The curve was calculated from the equation $Y = 193 - 145 \exp -0.0051\ X$, where Y is the metabolic response and X is the time in days.

Rats exposed to cold developed a striking increase in sensitivity to infused noradrenaline. These data suggested an asymptotic relationship of the form $Y = \alpha + \beta\rho^X$ where Y is the metabolic response and X is time in days. Maximum likelihood estimates, a, b, and r, of the parameters were calculated by a method described by Stevens (7). Analysis of covariance indicated as good a fit as could be expected, the residual variation about the fitted curve being of the same order as the variation between rats tested after the same period of exposure to cold. The values of the parameters were $a = 193 \pm 16$, $b = -145 \pm 19$, and $r = 0.95 \pm 0.03$. This equation can be set in the form $Y = 193 - 145 \exp -0.051X$ thus giving a period of approximately 14 days for development of half-maximal response.

The average initial colonic temperature of all rats was 35.5° C (range 33.1 to 37.1). The warm-acclimated rats showed an average decline in rectal temperature of −3.5° C during the 100 minutes of noradrenaline infusion while the cold-exposed rats showed changes of −2.1 to +3.6° C during the same time depending on the length of time of previous exposure to cold.

To assess the thermogenic influence of the change in environmental temperature, three warm- and three cold-acclimated rats were tested under similar experimental conditions but without noradrenaline infusion. The average metabolic response to the change in temperature of the environment (30° C to 20° C) was +12.3 in the warm- and −10.0 in the cold-acclimated rats.

These values were insignificant when compared with the large changes observed during noradrenaline infusion in the cold-exposed rats.

Calorigenic Response in Functionally Eviscerated Cold-acclimated Rats

The average oxygen consumption of the different groups of rats, eviscerated or sham-operated and infused with noradrenaline or not, are given in Fig. 4.

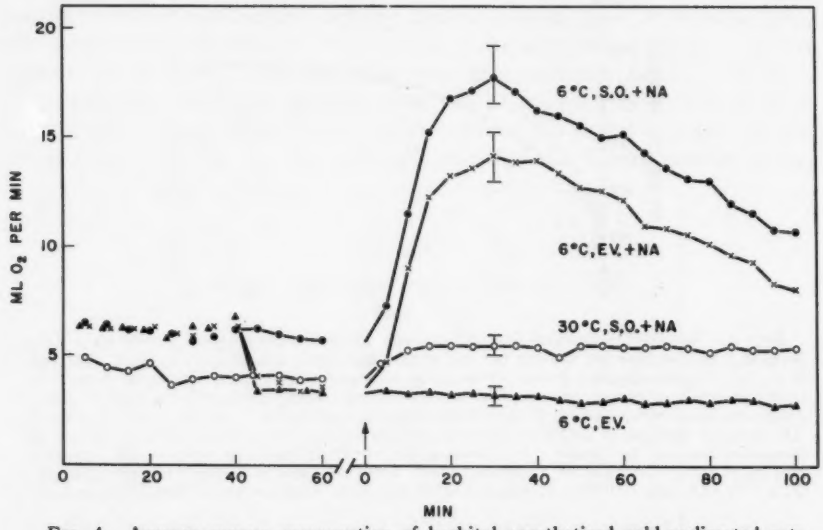

FIG. 4. Average oxygen consumption of barbital anesthetized cold-acclimated rats (three rats per group), functionally eviscerated and infused with 0.1% sodium bisulphite in saline (6° C, EV.), functionally eviscerated and infused with noradrenaline (in 0.1% sodium bisulphite in saline) at a dose of 1 μg per minute (6° C, EV. + NA), sham-operated and infused with noradrenaline at the same dose level (6° C, S.O. + NA). The average oxygen consumption of two warm-acclimated rats, sham-operated and infused with noradrenaline, is also given. Arrow indicates start of infusion which lasted 100 minutes. Vertical bars indicate total range of variation.

The two sham-operated warm-acclimated rats (30° C, S.O. + NA) gave little response to noradrenaline infusion while the similarly treated cold-acclimated rats (6° C, S.O. + NA) gave a large increase in oxygen consumption during infusion of the amine. Infusing noradrenaline into the eviscerated cold-acclimated rats (6° C, EV. + NA) also induced a marked rise in oxygen consumption. After evisceration the oxygen consumption of the cold-acclimated rats (6° C, EV.) fell to stable values which were maintained throughout the experiment. In the eviscerated and sham-operated rats infused with noradrenaline the increased O_2 consumption was not maintained as well as in non-operated rats otherwise similarly treated. No obvious explanation can be given for the alteration in the pattern of response of this group of rats.

In order to estimate the effect of evisceration on the response of cold-acclimated rats to noradrenaline infusion, the differences between average oxygen consumption before (45 to 60 minutes) and during noradrenaline infusion (25 to 40 minutes) were calculated. The sham-operated 6° C rats gave

a maximal increase of 11.7 ± 0.7 ml O_2 per minute and the functionally eviscerated 6° C rats gave a maximal increase of 10.3 ± 0.7 ml O_2 per minute, the difference between the two groups being non-significant.

Discussion

The three groups of warm-acclimated rats in this study manifested slightly different sensitivities to noradrenaline. One out of four rats in the first group showed an increase in O_2 consumption during noradrenaline infusion while all 13 warm-acclimated rats, in the second and third groups, gave a definite metabolic response with an infusion rate of 1 μg noradrenaline per minute. This difference was probably associated with the lower colonic temperatures of the first group of 30° C rats resulting from the colder initial environmental temperature used in these tests. The colonic temperatures of rats in the first group were in a range at which oxygen consumption becomes a function of body temperature (8). Presumably in the warm-acclimated rats at 20° C the small calorigenic effect of noradrenaline was balanced by the decrease in oxygen consumption due to body cooling. This would not be the case for warm-acclimated rats in the second and third sets of experiments.

The striking sensitivity of cold-acclimated rats to L-noradrenaline discovered by Hsieh and Carlson (1) is amply confirmed by the data given here. The calorigenic response was shown to be a function of the amount of noradrenaline infused per unit time and was found to be related to the time the animals had spent in the cold room. The time course of development of sensitivity to noradrenaline parallels other changes, related to heat production, which have been observed during development of cold acclimation such as increase in food consumption, increase in survival at lethal cold, increase in resistance to body cooling, and replacement of shivering by other means of heat production (9). In particular, the metabolic response to noradrenaline during acclimation bears a striking inverse resemblance to the curve of muscle electrical activity as a function of time of exposure to 6° C measured in unanesthetized rats. Muscle electrical activity was found to return to the basal level observed in rats at 30° C in about 4 weeks (3). This corresponds approximately to the time required for development of maximal sensitivity to noradrenaline noted in this study. Moreover, the magnitude of the metabolic response to infusion of the higher doses of noradrenaline is so large that it approaches values of oxygen consumption measured in intact rats at −25° C (the average oxygen consumption for 10 rats was 22.5 ml per minute (10)). These observations provide further evidence for the suggested role of noradrenaline as the mediator of non-shivering heat production in the cold-acclimated white rat (1). This conclusion is further supported by the observation that abdominal viscera are not essential for full manifestation of the noradrenaline effect in the cold-acclimated rats just as they were not necessary for manifestation of the metabolic response of the cold-acclimated rat to cold exposure (11). This last finding has now received support from Kawahata and Carlson (12), who could find no significant change in liver blood flow, no change in liver tissue oxygen tension, and no difference between liver and rectal temperatures when curarized

cold-acclimated rats increased their oxygen consumption by 50% on exposure to a cold environment.

Since both the cold- and the noradrenaline-induced increases in oxygen consumption in short-term experiments on cold-acclimated rats appear to be independent of the presence of the liver, other tissues should be considered as possible sites for non-shivering thermogenesis. The suggestion has previously been made that muscle could conceivably be the site of this extra heat production not associated with increase in tone or shivering (11). This hypothesis is appealing for many reasons. There is a large mass of tissue involved and its potential for very large increases in oxygen consumption is well known in work and even in the shivering warm-acclimated rat when in the cold. Demonstration of a direct action of 2,4-dinitrophenol on skeletal muscle (13) indicates the possibility of large increases in oxygen consumption without mechanical activity in this tissue.

Cottle and Carlson (4) have demonstrated that adrenal demedullation lowers the cold response of the curarized cold-acclimated rat and Hsieh *et al.* (14) have shown that sympatholytic and ganglionic blocking agents prevent this response. Both adrenal medulla and an intact sympathetic nervous system are then essential for full manifestation of non-shivering thermogenesis in the cold-acclimated rat. It would be necessary, therefore, to explain sympathetic involvement in the non-shivering thermogenic response of muscle, a tissue which has not been definitely shown to be directly innervated by sympathetic fibers (15). Identification of the site or sites of release of noradrenaline other than the adrenal medulla involved in the muscle response would also remain to be identified.

Acknowledgment

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PREPARATION AND PROPERTIES OF TRANSKETOLASE FROM PORK LIVER[1]

F. J. SIMPSON

Abstract

Transketolase of pork liver has been purified 90-fold and separated from ribulose 5-phosphate 3-epimerase. The transketolase is most stable between pH 7.5 and 8.5 and below 40° C. The pH range for optimum activity is between 7.6 and 8.1. Activation by magnesium ions or thiamine pyrophosphate could not be demonstrated, but thiamine pyrophosphate increased the stability of the enzyme. Sulphydryl agents, such as *p*-chloromercuriphenyl sulphonic acid and *N*-ethylmaleimide, and heavy metal ions, such as cupric, mercuric, and zinc, at relatively high concentrations inhibited the enzyme.

Introduction

Transketolase is widespread in nature and has been purified from spinach, yeast, and rat liver. During studies on the purification of the D-xylulokinase of *Aerobacter aerogenes* and separation from 3-epimerase* we required a preparation of transketolase free of epimerase. Such a preparation can be obtained from rat liver (1, 2), but this was not a convenient source. Attempts to obtain a preparation from yeast and spinach failed even though spinach from a different clime had previously been suitable (3). Subsequently, Srere *et al.* (4) published a procedure for removing the epimerase from yeast transketolase. In the meantime, transketolase was found to be present in pork liver, and in lesser amounts in beef liver. Both could readily be obtained from the local abattoir. In this paper a procedure is described for preparing from pork liver a preparation of transketolase that is free of 3-epimerase. Some properties of the enzyme are also described.

Materials

R-5-P was purchased from Schwarz Laboratories Inc., Mount Vernon, N.Y., and the pyridine nucleotides from Sigma Chemical Company, St. Louis, Mo. Xu-5-P was prepared by phosphorylating D-xylulose with the kinase obtained from *Aerobacter aerogenes* (5). S-7-P was prepared as previously described (6). Isomerase was prepared by the method of Hurwitz *et al.* (7) and 3-epimerase (ammonium sulphate II) by the method of Hurwitz and Horecker (8). Hexose phosphate isomerase, glucose 6-phosphate dehydrogenase, and transaldolase were the same preparations as used previously (6). Glycerol phosphate dehydrogenase was a crude preparation from rabbit muscle that also contained triose phosphate isomerase and aldolase, but was free of 3-epimerase (6).

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*The following abbreviations are used: G-3-P, D-glyceraldehyde 3-phosphate; R-5-P, D-ribose 5-phosphate; Ru-5-P, D-ribulose 5-phosphate; Xu-5-P, D-xylulose 5-phosphate; F-6-P, D-fructose 6-phosphate; S-7-P, D-sedoheptulose 7-phosphate; DPN, diphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide; DEAE, diethylaminoethyl cellulose; TEA, triethanolamine; TRIS tris(hydroxymethyl)aminomethane; versene, disodium ethylene diaminetetraacetate; 3-epimerase, D-ribulose 5-phosphate 3-epimerase; isomerase, D-ribose 5-phosphate isomerase.

DEAE, purchased from Brown Corporation, Berlin, New Hampshire, U.S.A., was equilibrated with 0.02 *M* TRIS – HCl 0.005 *M* $MgCl_2$ buffer (pH 7.5). Fifty grams of the cellulose was mixed with 3 liters of buffer and filtered on a fritted glass funnel. The process was repeated until the pH of the filtrate was 7.5. The DEAE was then rinsed with glass-distilled water, and could, if desired, be dried at room temperature and stored. DEAE was reclaimed after use by first washing by decantation with 4 *M* NaCl, followed by repeated washings with 1 *N* NaOH until the supernatants were no longer colored. The cellulose was then suspended in sufficient 1 *N* HCl to make a strongly acid suspension, quickly filtered on a Büchner funnel, suspended once more in 1 *N* NaOH, and the alkali removed by washing with water. This reclaimed DEAE, after equilibration with TRIS – $MgCl_2$ buffer, performed as well as, if not better than, the original material.

Methods

Analytical

Spectrophotometric measurements were made at room temperature with a Beckman DU spectrophotometer in cuvettes with a 1-cm light path and a capacity of 1.5 ml. Transketolase activity was assayed by the method of Horecker *et al.* (9) or alternatively in the absence of isomerase and 3-epimerase, with Xu-5-P as the substrate and R-5-P as the acceptor (Fig. 1).

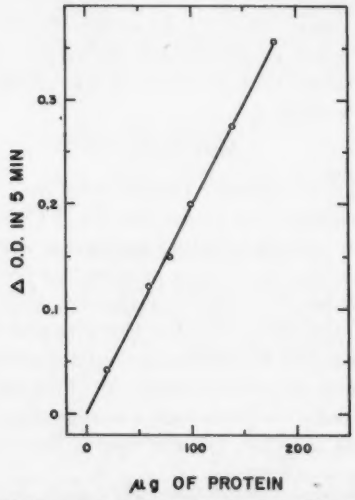

FIG. 1. Assay method for transketolase with Xu-5-P. The reaction mixture (volume = 1.0 ml) contained 70 μmoles TEA buffer (pH 7.8), 5 μmoles versene (pH 7.8), 0.09 μmole DPNH, 1.0 μmole R-5-P, 0.3 μmole Xu-5-P, and 1 mg of the glycerophosphate dehydrogenase preparation. A unit of enzyme is defined as that which causes a change in optical density of 1.000 per minute at room temperature calculated from the period of linear reaction rate.

Transketolase activity was also determined by measuring the amount of S-7-P produced in 10 minutes at 30° C. The solution (volume = 1 ml) con-

tained 20 μmoles TEA buffer (pH 7.8), 1 μmole Xu-5-P, 2 μmoles R-5-P, and from 0.04 to 0.2 unit of transketolase. The reaction was stopped by the addition of 0.1 ml of 1.0 *N* HCl and the tube was placed in ice. Just before analysis for the S-7-P produced (10), the mixture was neutralized by the addition of 0.1 ml of 1 *N* NaOH.

DPNH oxidase was determined in a reaction mixture of 1 ml consisting of water, enzyme, 20 μmoles of TEA buffer (pH 7.8), and 0.06 μmole of DPNH. Likewise, the activity of alcohol dehydrogenase was measured in a reaction mixture (volume = 1 ml) containing 20 μmoles of TEA buffer (pH 7.8), 0.06 μmole of DPNH, and 0.4 μmole of propionaldehyde. The activity of 3-epimerase was measured in the presence of isomerase and transketolase (9). All activities were expressed as that amount of enzyme which caused a change in optical density of 1.000 per minute, calculated from a 5-minute period when the rate was linear.

Protein was estimated by the method of Lowry *et al.* (11) except that the time required to develop the color was reduced by placing the mixture of sample and alkali in a water bath at 37° C for 5 minutes and then for an additional 10 minutes after the phenol reagent was added. S-7-P was determined spectrophotometrically with transaldolase and Xu-5-P with transketolase (10).

Purification of Transketolase

Unless otherwise stated, all operations were performed at 0 to 5° C. Precipitates were separated by centrifugation at 10,000×*g* in a Lourdes L centrifuge or at 20,000×*g* in a Servall SS1 centrifuge. The enzyme is inactivated if the pH falls much below neutrality.

Fresh pork liver was obtained at the local abattoir and cooled in ice. Once chilled, the liver may be stored for 18 hours without affecting the yield of transketolase. The liver was then cut into strips and 400 g homogenized for 2 minutes with 1600 ml of cold acetone (−20° C) in a Waring blendor (1-gal capacity). The homogenate was filtered quickly under vacuum through No. 230 Reeve Angel paper in 18-cm Büchner funnels. The filter paper was peeled off the semidry pulp and the homogenizing process repeated with a second 1600-ml portion of cold acetone. The pulp was then dried under vacuum over calcium chloride. The resultant material should be dry and friable, but if not, a third treatment with acetone will produce the desired product. When stored at 0° C under vacuum, the acetone powder retains its transketolase activity for months. One preparation stored for 9 months contained 80% of its original activity.

The transketolase was extracted by suspending 50 g of the acetone powder in 500 ml of 0.015 *M* Na_2CO_3 and slowly stirring for 45 minutes at room temperature. The extract (pH 8.0) was then centrifuged at 10,000×*g* for 5 minutes in the cold to remove the insoluble debris (Table I). The red supernatant solution can be stored at 2° C for 24 hours without loss in activity.

Twelve grams of DEAE, previously equilibrated and dried, was whipped into an aqueous suspension and allowed to settle, preferably overnight at 0° C. The excess water was then removed by decantation or by vacuum filtration on

TABLE I

Purification of transketolase from pork liver

Step	Volume, ml	Total activity, units	Specific activity*	Purification, fold	Yield, %
Crude	450	470	.03	1	
DEAE	800	460	.05	1.7	98
Heat, 60° C	800	425	.05	1.7	90
Lead acetate	850	410	.30	10	87
Acetone	100	400	.72	24	85
$CaPO_4$ gel	45	320	1.55	52	68
$(NH_4)_2SO_4$	8	150	1.40	47	32
DEAE	15	140	2.69	90	30

*Units per milligram of protein.

a coarse fritted glass funnel. The DEAE was then added to the crude extract (pH 8.0) and thoroughly mixed for 5 minutes. The DEAE was removed by vacuum filtration on a fritted glass funnel and rinsed in the funnel with 25 ml of water. The filtrate was then treated in the same fashion with a second batch of DEAE (12 g). The filtrate contains most of the transketolase, but only about 1% of the initial amount of 3-epimerase and of DPNH oxidase. The 3-epimerase can be eluted from DEAE with 3 *M* NaCl.

The filtrate, at pH 8.0, was transferred to a 2-liter beaker that was then placed in a boiling-water bath. The enzyme preparation was stirred until the temperature reached 60° C (3.5 minutes) when the beaker was plunged into a dry ice – ethanol bath and the enzyme rapidly cooled to 0° C. The heat treatment usually coagulates some protein that is difficult to remove by centrifugation, thus the next step is performed without removing this material. Occasionally a heavy voluminous precipitate forms and this is removed by centrifugation before proceeding with the next step.

A 20% aqueous suspension of basic lead acetate was mixed for 10 minutes and allowed to settle at room temperature overnight. The clear supernatant was used. (A trial experiment should be performed to determine how much of the basic lead acetate may be added without precipitating transketolase.) For each milliliter of the enzyme preparation at pH 8.0, 0.1 ml of the solution of basic lead acetate was added slowly and the mixture stirred for 5 minutes. The voluminous precipitate was separated by centrifugation at $10,000 \times g$ for 5 minutes. Then 0.15 g of $NaHCO_3$ per ml of basic lead acetate solution used was added to the supernatant solution slowly with stirring, mixed for 5 minutes, and the lead carbonate removed by centrifugation.

The clear supernatant was adjusted to pH 8.0 and cooled to 0° C in a dry ice – ethanol bath. One volume of cold acetone (−20° C) was added slowly while the temperature was maintained at 0° C. Stirring was continued for another 3 minutes. The pink precipitate was recovered by centrifugation for 2 minutes at $10,000 \times g$ and dissolved in 100 ml of 0.001 *M* TEA – 0.001 *M* cysteine buffer (pH 9.5). The pH of the enzyme preparation was then adjusted to 8.0 and any insoluble material was removed by centrifugation.

Calcium phosphate gel (50 ml, 15 mg per ml) was added to the solution and mixed for 5 minutes. The gel was recovered by centrifugation and washed

with 200 ml of cold 0.05 *M* $NaHCO_3$. The enzyme was eluted by treating the gel twice with 20 ml of 0.125 *M* phosphate – 0.001 *M* cysteine buffer (pH 8.0).

One and one-tenth volumes of cold, saturated ammonium sulphate (neutralized with NH_4OH so that a 1:5 dilution gave a pH of 7.5) was added to the pooled eluates and the precipitate removed by centrifugation at 20,000×*g* for 5 minutes. Solid ammonium sulphate was then added to raise the concentration to 70% of saturation. The precipitate was recovered by centrifugation at 20,000×*g* for 10 minutes and dissolved in 0.1 *M* TEA (5 ml). This preparation (pH 8.0) was then dialyzed overnight against cold, flowing 0.002 *M* TEA.

One gram of DEAE, previously equilibrated and dried, was suspended in water and the excess water removed by filtration under vacuum. The moist DEAE was then added to the dialyzed enzyme preparation and mixed thoroughly with a spatula. Another gram of DEAE was suspended in cold water and the excess water removed on a fritted glass funnel (diameter = 23 mm). The mixture of enzyme and DEAE was then placed on top of this moist cake of DEAE and the enzyme recovered by filtration under vacuum. The cellulose was rinsed twice with 1 ml of water. Solid glycylglycine to a molarity of 0.20 and thiamine pyrophosphate to a molarity of 0.01, together with sufficient sodium hydroxide to maintain a pH of 8.0, were then added. The preparation was stored at −20° C.

Whereas the crude extract of the acetone powder contained isomerase, 3-epimerase, transaldolase, glucose 6-phosphate dehydrogenase, glycerol phosphate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, xylulokinase, DPNH oxidase, and alcohol dehydrogenase, the purified preparation was free from these enzymes. The purified enzyme had some aldolase activity. Initially, the purification procedure did not contain the step employing basic lead acetate and the final preparation was rich in alcohol dehydrogenase. This enzyme was found to be inactivated by high dilution in water, but was stable when diluted in phosphate buffer. In addition, the dehydrogenase was not inhibited by 2×10^{-4} *M* iodoacetamide or *N*-ethylmaleimide, but, as is typical of alcohol dehydrogenases, was completely inhibited by 1×10^{-4} *M* *p*-chloromercuriphenyl sulphonic acid and was sensitive to heavy metals. Since liver transketolase was less sensitive to heavy metals, the alcohol dehydrogenase could be conveniently removed by means of basic lead acetate.

Experimental

Absorption and Elution Studies with Calcium Phosphate Gel

In attempting to devise a procedure that would separate transketolase and 3-epimerase, we observed that transketolase and half of the 3-epimerase could be absorbed by calcium phosphate gel from the crude extract of the acetone powder. The absorbed 3-epimerase could then be removed, together with 10% of the transketolase, by repeatedly washing (4 to 5 times) the gel with 0.05 *M* sodium bicarbonate buffer (pH 7.6). The pooled washings also contained about 40% of the absorbed protein and most of the colored material. Transketolase, purified 2- to 3-fold, could then be eluted with 0.2 *M* potassium phosphate buffer (pH 8.0), or with 0.5 *M* carbonate – 0.2 *M* versene buffer (pH 8.0). This

process, however, was tedious and time consuming and therefore abandoned in favor of separation by DEAE.

During this work we had an opportunity to compare the efficiency of calcium phosphate gels that were freshly prepared, aged for 1 month, 12 months, and 18 months. All four gels were prepared by the same person under similar conditions and were stored at concentrations of 40 to 50 mg per ml at 2° C (12). No difference in performance was observed between the freshly prepared gel and that 1 month old, whereas the gels 12 and 18 months old were less effective. The newer gels absorbed more protein and more enzyme per milligram of gel and they permitted a greater purification than did the older gels (Table II).

TABLE II

Comparison of the absorption and elution of transketolase from new* and old* calcium phosphate gels

Treatment	Enzyme, %		Protein, %		Specific activity	
	New	Old	New	Old	New	Old
Original	100	100	100*	100	0.033	0.033
Absorbed on 120 mg gel	76	59	42	30	—	—
Eluted with 0.05 *M* HCO_3†	5	9	5	8	0.032	0.035
0.05 *M* PO_4†	21	27	15	10	0.042	0.092
0.1 *M* PO_4†	38	15	10	6	0.115	0.080
0.2 *M* PO_4†	20	10	7	4	0.090	0.075

*New = freshly prepared. Old = stored at 2° C for 1 year.
†The eluting agents consisted of sodium bicarbonate, or potassium phosphate buffers at pH 8.0.

Kornberg and Horecker (13), likewise, have indicated that freshly prepared calcium phosphate gel gives better results in the purification of glucose 6-phosphate dehydrogenase than aged gels.

Stability

The crude extract has been stored at 0° C for 2 days and at −20° C for 1 week with negligible loss in transketolase activity. The purified preparation at pH 7.8 to 8.0 is likewise relatively stable and has been stored for 3 months at −20° C with little loss. Preparations that after prolonged storage appear to have lost 5 to 10% of their activity may be restored by incubating for a few minutes in 0.01 *M* cysteine (pH 7.8) (1). Pork liver transketolase is most stable between pH 7.5 and 8.5 (Fig. 2). At pH 7.8, the enzyme is relatively stable below 40° C, but is rapidly inactivated at higher temperatures (Fig. 2).

Optimum pH

The optimum pH range for activity, as determined by both the spectrophotometric method and by measurement of the amount of S-7-P produced from Xu-5-P and R-5-P, is 7.6 to 8.1 (Fig. 3).

Effect of Activators and Inhibitors

Activation by magnesium ions or by thiamine pyrophosphate could not be demonstrated even after dialysis for 5 days in cold, flowing 0.6% versene – 0.9% KCl (pH 7.5) (14, 15). Attempts to dissociate the coenzyme with acid ammonium sulphate (1) resulted in complete destruction of the enzyme. Thiamine pyrophosphate, however, was found to stabilize the enzyme. When 1 ml of

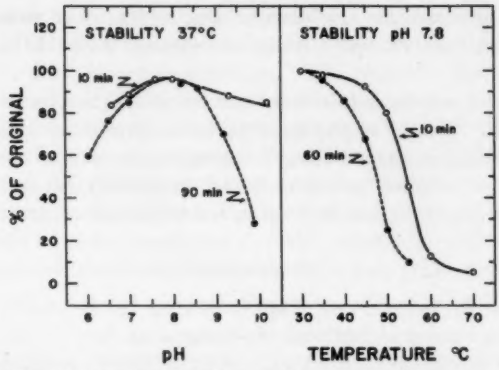

FIG. 2. Stability of liver transketolase when held at different pH values and at different temperatures. One unit of liver transketolase incubated in 0.5 ml of 0.1 *M* TEA – 0.1 *M* $NaHCO_3$ buffer. In determining stability at different pH values, 0.1-ml aliquots were removed and added to 0.2 ml of cold 0.2 *M* TEA buffer (pH 7.8). The remainder was used to determine the pH. In determining stability at different temperatures, 0.1-ml aliquots were removed at intervals and added to 0.1 ml of cold 0.02 *M* TEA buffer (pH 7.8).

transketolase at pH 7.8, containing 10 μmoles of thiamine pyrophosphate, was held for 18 hours at 30° C, all of the activity was retained as compared to 80% for that containing 250 μmoles of glycylglycine and 70% for the control. As a result of these observations, the purified transketolase preparations were stored in 0.01 *M* thiamine pyrophosphate.

Cysteine, glutathione, glycylglycine, TRIS, sodium bicarbonate, and sodium chloride were innocuous when added to the spectrophotometric assay system in concentrations of 1, 10, and 20 μmoles per ml. Whereas at a concentration of 1 μmole per ml arsenate, borate, magnesium chloride, phosphate, phosphite,

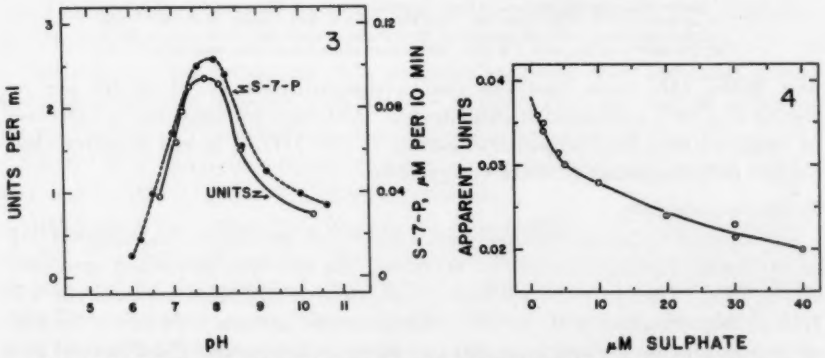

FIG. 3. The effect of pH on the activity of transketolase. The units of activity were determined in the standard reaction mixture at room temperature as described in Fig. 1 except that the triethanolamine buffer was fortified with 20 μmoles of sodium bicarbonate. The effect of pH on the production of sedoheptulose was determined at 30° C with 0.08 unit of transketolase in a reaction mixture containing 0.1 *M* TEA – Na_2CO_3 buffers.

FIG. 4. The effect of ammonium or sodium sulphate on the determination of transketolase. The reaction mixture contained 1.03 μmoles of sulphate that arose from the enzyme preparations.

pyrophosphate, perchlorate, trichloroacetate, and versene were innocuous, at 20 μmoles per ml they caused a decrease between 6 and 15% in the rate of oxidation of DPNH.

Ammonium and sodium sulphate had an equal and marked depressing effect on the rate of the reaction in the spectrophotometric assay (Fig. 4). This was particularly irksome in determining 3-epimerase for in the assay with R-5-P and isomerase, ammonium sulphate could completely mask the presence of epimerase. Such fractions had to be dialyzed before an accurate determination could be made. The inhibitory effect of sulphate is due to both inhibition of transketolase (Table III) and of glycerophosphate dehydrogenase, for Sellinger

TABLE III

Inhibition of transketolase

Inhibitor	Concentration, molarity	Inhibition, %
p-Chloromercuriphenyl sulphonic acid	2.5×10^{-5}	6
	2.5×10^{-4}	16
Iodoacetamide	5×10^{-3}	20
N-Ethylmaleimide	1×10^{-4}	3
	1×10^{-3}	20
Copper acetate	5×10^{-5}	7
	2×10^{-4}	19
	2×10^{-3}	50
Mercuric chloride	5×10^{-6}	7
	2×10^{-4}	40
	8×10^{-4}	72
Zinc chloride	2×10^{-3}	8
Lead acetate	2×10^{-3}	40
Potassium phosphate	2×10^{-2}	10
Sodium sulphate	2×10^{-2}	7

NOTE: The reaction mixture (1.0 ml) contained inhibitor, 0.2 unit of transketolase, 20 μmoles of TEA buffer (pH 7.8), 2 μmoles of R-5-P, and 1 μmole of Xu-5-P. The reaction was begun with the addition of Xu-5-P and stopped after 10 minutes at 30° C by the addition of 0.1 ml of 1 *N* HCl. Neutralized aliquots were then assayed for S-7-P.

and Miller (16) have observed that a concentration of 63 μmoles per ml results in a 95% inhibition of this enzyme. Although transketolase is inhibited by reagents that bind sulphydryl groups (Table III), it is less sensitive than alcohol dehydrogenase to these compounds.

Substrate Specificity

In the spectrophotometric assay with Xu-5-P as the donor, and either R-5-P or erythrose 4-phosphate as the acceptor, the reaction proceeded smoothly and quickly. The rate obtained with glycolaldehyde and DL-glyceraldehyde was 1/50 of that obtained with R-5-P. Aldopentoses, hexoses, and formaldehyde, as well as glucose 6-phosphate, did not serve as acceptors. F-6-P served as a donor in the presence of R-5-P (6), but D- and L-ribulose, D-xylulose, D-fructose, and sedoheptulose did not form S-7-P in the presence of R-5-P.

Equilibrium

The equilibrium point was estimated by incubating Xu-5-P and R-5-P with transketolase at 25° C and determining the amounts of Xu-5-P, G-3-P, and

S-7-P by enzymatic assays when equilibrium was attained. The reaction mixture (1.0 ml) consisted of 1 μmole of Xu-5-P, 1 μmole of R-5-P, 20 μmoles of TEA buffer (pH 7.8), and transketolase. The reaction was stopped by the addition of 0.1 ml of *N* HCl and, after neutralization, aliquots were removed for analysis. Equilibrium was attained within 30 minutes with 0.7 unit of transketolase, when the reaction mixture was found to contain 0.57 μmole of Xu-5-P (average of three determinations), 0.36 μmole of G-3-P, and 0.40 μmole of S-7-P. At equilibrium the ratio of Xu-5-P to S-7-P is approximately 1:5.

Discussion

The transketolases of rat liver and pork liver appear to be quite similar: neither require Mg^{++}, both are apparently most stable around pH 8.0, relatively stable to organic solvents, and, after storage for long periods of time at −20° C, can be reactivated by cysteine. Pork liver is apparently a richer source of 3-epimerase than rat liver for when the purification procedure devised for rat liver (1) was applied to pork liver, the resultant preparation was still rich in 3-epimerase, whereas rat liver yielded a preparation free from 3-epimerase (17).

The four transketolases that have been purified and studied are apparently most stable and most active at pH values around 7.4 to 8.0 (1, 15). Transketolase of pork liver is denatured at low pH values while that of spinach is more tolerant and can be partially recovered after treatment at pH 2.2 with ammonium sulphate (1). All four cleave Xu-5-P, S-7-P, and F-6-P, transferring the glycolaldehyde fragment to a suitable acceptor (1, 15, 18, 19). The transketolase of pork liver appears to be more specific than that of yeast. Such compounds as DL-glyceraldehyde, glycolaldehyde, and formaldehyde will not serve as suitable acceptors for pork liver transketolase but do for yeast (15, 20, 21, 22).

Axelrod and his colleagues (23) obtained evidence that indicated transketolase of spinach was inhibited by 10^{-2} *M* phosphate and 10^{-4} *M* *p*-chloromercuribenzoate. Transketolase of pork liver is less affected by such agents. 2.5×10^{-4} *M* *p*-chloromercuriphenyl sulphonic acid caused only a 16% inhibition and at 10^{-2} *M* phosphate a 10% inhibition.

Although a requirement for thiamine pyrophosphate could not be demonstrated, the coenzyme appears firmly bound to the apoenzyme and separation, as indicated by the enhanced stability of transketolase in the presence of thiamine pyrophosphate, may result in complete inactivation, thus making it difficult to demonstrate a requirement.

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GLYCERIC ACID KINASE ISOLATED FROM A POLISH VARIETY OF RAPESEED (BRASSICA CAMPESTRIS L.)[1]

K. OZAKI[2] AND L. R. WETTER

Abstract

Glyceric acid kinase from Polish variety rapeseed (*Brassica campestris* L.) was purified approximately 30-fold by ammonium sulphate fractionation and adsorption on alumina C_γ gel. The enzyme has a pH optimum at 7.9 and a pH stability range extending from 6.5 to 7.5. The maximum temperature for the reaction was 45° C. The phosphorylation required ATP and a metal ion; Mg^{++} was slightly more effective than Mn^{++} and Co^{++}, while Ni^{++} and Zn^{++} were ineffective. The Michaelis constants for the Mg–ATP complex and the substrate were 7.3×10^{-4} *M* and 1.6×10^{-3} *M* respectively. The reaction products, ADP and 3-phosphoglyceric acid, inhibited the phosphorylation. Sulphydryl reagents such as *p*-chloromercuribenzoate, *o*-iodosobenzoate, N-ethylmaleimide, and iodoacetate completely inhibited the enzyme at low concentrations. 3-Phosphoglyceric acid was isolated and characterized from the enzyme reaction mixture.

Introduction

Glyceric acid and its phospho-derivative(s) are widely distributed in nature. They have been found not only in mammalian tissue but also in plant material, e.g. in barley leaves (1), red algae (2), and in potatoes (3). The enzymatic phosphorylation of D-glyceric acid was demonstrated in yeast by Black and Wright (4) and in mammalian liver by two other groups of workers (5, 6). ATP[3] was the phosphate donor in every case investigated.

Recently Ichihara and Greenberg (7) reported that serine and phosphoserine were derived from glyceric acid. Higher plants also have the ability to form phosphoserine from 3-PGA (8). Since there appears to be no direct phosphorylation of serine (9), glyceric acid may be of importance in the biosynthesis of phosphoserine and serine. In addition since phosphoserine arises in plants from 3-PGA, one can suggest that the enzymatic phosphorylation of glyceric acid might occur in plants.

This paper describes the identification, partial purification, and some of the properties of a glyceric acid kinase found in rapeseed.

Methods and Materials

Free DL-glyceric acid was obtained by converting the commercial calcium salt to the sodium salt with sodium carbonate and then passing it through an Amberlite IR-120 column. The concentration of glyceric acid was determined with chromatropic acid (10). The hydrogen ion concentration was adjusted to pH 7 with sodium hydroxide before use in the enzyme assay. ATP was a crystalline sodium salt obtained from Pabst Laboratories. 3-PGA was obtained

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Issued as N.R.C. No. 5492.

[2]Postdoctorate Fellow, National Research Council of Canada, 1957–1959.

[3]The following abbreviations are used: ATP, adenosine triphosphate; ADP, adenosine diphosphate; 3-PGA, 3-phosphoglyceric acid; GSH, reduced glutathione; EDTA, ethylenediamine tetraacetic acid; TCA, trichloroacetic acid; TPN, triphosphopyridine nucleotide.

from Nutritional Biochemicals Corporation, as the barium salt. Other chemicals employed in this investigation were commercial products unless otherwise indicated.

Protein was determined according to the method of Lowry *et al.* (11). 3-PGA was isolated and identified by a method used by Ichihara and Greenberg (5).

Experimental

Estimation of Enzyme Activity

Enzymatic activity was determined by the manometric method of Colowick and Kalckar (12). Unless otherwise stated the main compartment of the Warburg flask contained 12 μmoles ATP, 12 μmoles of $MgCl_2$, 5 μmoles of GSH, 0.1 ml 0.4 *M* $NaHCO_3$, and enzyme to a total volume of 0.9 ml. Glyceric acid (10 μmoles in 0.1 ml) was placed in the side arm of the flask. The flasks were then flushed with a 95% N_2 and 5% CO_2 gas mixture[4] for 10 minutes followed by a 30-minute equilibration period. The enzymatic reaction was initiated by tipping the glyceric acid from the side arm into the main compartment. The gas released was read at 10-minute intervals for at least 60 minutes. The temperature of the reaction was 30° C. Appropriate controls and corrections were always employed.

The enzyme under conditions of the assay gave a zero-order reaction (Fig. 1). This order was maintained for at least a 2-hour period and different enzyme concentrations also gave the same order. Figure 1 shows that about 25% of the D-glyceric acid present was phosphorylated in 120 minutes. In the enzyme range used for the investigation, the microliters of CO_2 released was proportional to the enzyme concentration (Fig. 2). This relationship prevailed for either the 1- or 2-hour reaction times. The 60-minute time period was employed for this investigation primarily because it shortened the assay considerably.

One unit of glyceric acid kinase is defined as the amount of enzyme that will catalyze the phosphorylation of 1 μmole of D-glyceric acid (5) in the first 60 minutes at 30° C for the conditions stated above.

Partial Purification of Enzyme

The seeds of Polish variety rapeseed (*Brassica campestris* L.) were ground in Skellysolve F (b.p. 35–58° C) in a Waring blendor, filtered, washed free of oil with solvent, and air-dried. The meal was kept at 2° C and all operations were performed at the same temperature.

Sixty grams of rapeseed meal was homogenized in 600 ml of 0.2 *M* borate – 0.001 *M* EDTA buffer (pH 7.4) for 5 minutes in a "VirTis" homogenizer at a speed of 40,000 r.p.m. The homogenate was centrifuged and the supernatant adjusted to pH 7.0 with 1 *N* NaOH. The extract was then dialyzed against two changes of 0.05 *M* borate – 0.00025 *M* EDTA buffer (pH 7.6) for a period of 7 hours. The dialysis was necessary because prior to this operation addition of ATP to the original extract released large quantities of CO_2, thus making it impossible to assay the extract for enzyme. The dialyzate was designated as the crude extract (see Table I).

[4]Obtained from Linde Air Products Company, Toronto 7, Ontario.

μl CO_2 RELEASED

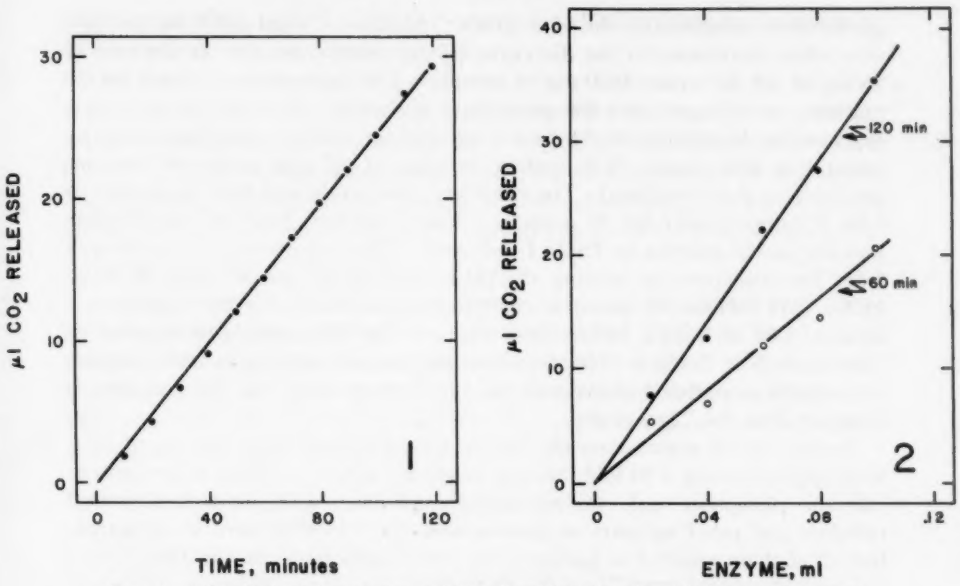

FIG. 1. The effect of time on the activity of purified glyceric acid kinase. The experimental conditions were those described in the text. The protein concentration was 66 μg per assay.

FIG. 2. The effect of varying enzyme concentrations on its activity. The assay was performed as described in the text. The enzyme concentration was 0.83 mg of protein per ml.

TABLE I

The purification of glyceric acid kinase of rapeseed

	Units*	Specific activity†
Crude extract	2860	0.34
Ammonium sulphate (40% to 60% saturation)	1775	0.52
Alumina Cγ, 1st eluate	705	5.26
Alumina Cγ, 2nd eluate	426	10.98

*Units as described in the text.
†Units per milligram of protein.

Three hundred and twenty milliliters of saturated ammonium sulphate (adjusted to pH 7) was added dropwise to 480 ml of crude extract over a period of 40 minutes. The solution was stirred for 1 hour and then centrifuged. The precipitate representing the 0 to 40% ammonium sulphate fraction was discarded. The 40 to 60% fraction was obtained by again adding dropwise 385 ml of saturated ammonium sulphate to 770 ml of the previous fraction (0 to 40% saturated ammonium sulphate). The solution was stirred for 2 hours and the precipitate was gathered by centrifugation. The precipitate was dissolved in 50 ml of 0.05 *M* borate – 0.00025 *M* EDTA buffer and dialyzed against the same buffer overnight. The final volume of the dialyzate was 71 ml.

The dialyzate was further fractionated by adsorption on alumina Cγ (13). Since concentrations are frequently very important in the success of this

purification considerable detail is given. Alumina Cγ gel (32.6 mg per ml) was added batchwise to the dialyzate (20 mg protein per ml) at the rate of 80 mg of gel for every 1000 mg of protein. The mixture was stirred for 20 minutes, centrifuged, and the precipitate discarded. The process was then repeated by the addition of the same amount of gel, stirred, centrifuged, and the precipitate was saved. Still another addition of gel was made and the two precipitates were combined. The combined precipitate was first suspended in 0.05 *M* borate buffer for 20 minutes. This treatment removed considerable non-enzymatic protein as Table I indicates. The enzyme was then removed from the adsorbent by stirring the gel in 0.03 *M* phosphate – 0.07 *M* NaCl buffer (pH 7.4) for 20 minutes, centrifuging, and dialyzing the supernatant against 0.05 *M* borate buffer for 3 hours. The dialyzate is designated as "1st eluate" in Table I. The dialyzate was treated once again with alumina Cγ exactly as outlined above and the supernatant from this fractionation is designated as the "2nd eluate".

Table I, which summarizes the results, shows that the yield was about 15% with approximately a 30-fold increase in specific activity. Other adsorbents as calcium phosphate gel, carboxymethyl cellulose, and diethylaminoethyl cellulose and other solvents as acetone and ethyl alcohol were investigated, but all of them resulted in low recovery and in only slight purification.

Enzyme Stability

The purified enzyme was reasonably stable when kept in the frozen state. Approximately 25% of the activity was lost over a period of 2 weeks when stored at pH 7.6 and at −12° C. The enzyme was completely inactivated in 1 hour at room temperature at pH 4.6 and 10.0. Under the same conditions only 70% of the activity was lost at pH 5.3 and 50% at pH 8.9. The kinase is most stable between pH 6.5 and 7.5.

Effect of pH and Temperature

The pH optimum was quite sharp and maximum activity was obtained at pH 7.9 (Fig. 3). The pH range over which the activity of the enzyme was examined was limited because of the $NaHCO_3$ buffer used in the assay. The

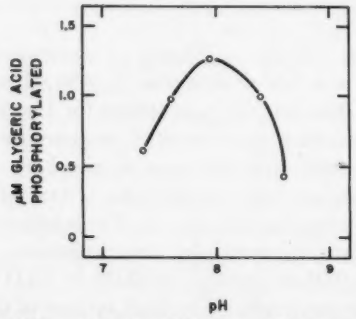

FIG. 3. The effect of pH on glyceric acid kinase. The experimental conditions were the same as those described in the text, except that changes in $NaHCO_3$ were employed to obtain varying pH's. The protein concentration was 95 μg per assay.

pH range in which optimum activity occurred is closely related to the range in which maximum stability was obtained.

The maximum temperature for the reaction is approximately 45° C. The amount of glyceric acid phosphorylated is doubled between 30 and 45° C. A plot of the log reaction rate against the reciprocal of the absolute temperature (20–45° C) gave a linear relationship, indicating that the energy of activation is independent of temperature. A value of 8500 calories per mole was obtained for the energy of activation and this is the same as found for choline kinase (9).

Effect of Mg^{++} *and ATP on Enzyme Activity*

A previous study (9) on choline kinase indicated that maximum phosphorylation was achieved when the ratio of Mg^{++} to ATP was 1 to 1. The phosphorylation of glycerate also requires a constant ratio of these two compounds (see Fig. 4); however, the range is not as narrow as for choline kinase. The ratio (Mg^{++}:ATP) varies from 2:1 to 1:1, and is not as critical for glyceric acid kinase as it is for choline kinase.

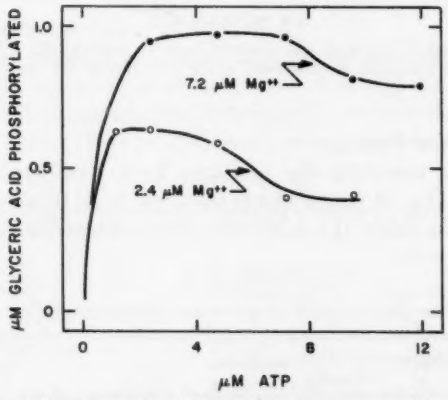

FIG. 4. The effect of varying the ATP and Mg^{++} concentrations. The experimental conditions were the same as described in the text except for the variations in ATP and Mg^{++} concentration.

The Michaelis constant for the Mg–ATP complex was calculated by the method of Lineweaver and Burk (14) by treating the complex as a substrate. This constant was calculated from velocity data obtained by changing the Mg–ATP concentration but maintaining a constant ratio (1:1). A straight line was fitted to the data and a constant of 7.3×10^{-4} M was obtained.

Effect of Glyceric Acid Concentration

The effect of glyceric acid on the reaction is shown in Fig. 5 for the conditions employed in the assay; approximately 10 μmoles of glyceric acid per test was required for maximum phosphorylation. The Michaelis constant was estimated to be 1.6×10^{-3} M, which is slightly lower than that found for choline kinase (9).

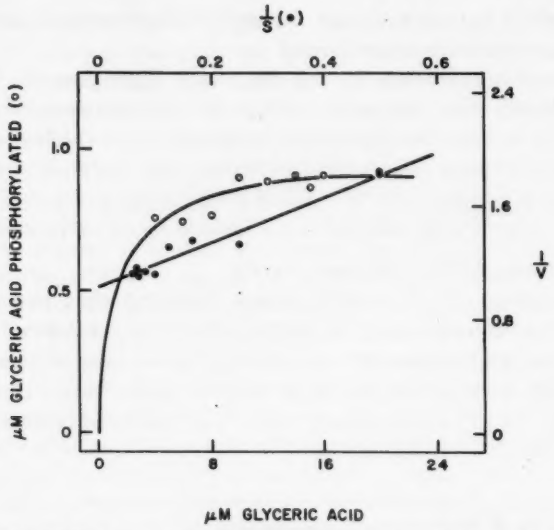

FIG. 5. The effect of varying the glyceric acid concentration. The conditions for the experiment were the same as described in the text except for the amounts of glyceric acid employed.

The Effect of Reaction Products

In most enzyme reactions the products have considerable effect on the over-all reaction. One of the products obtained is ADP and its effect on the reaction is shown in Table II. ADP definitely reduces the over-all amount of

TABLE II

The effect of ADP on glyceric acid kinase activity

Additions to Warburg flask, μmoles		Relative activity, %
ADP	Mg^{++}	
0	6	100
0.5	6	103
1	6	96
2	6	77
4	6	75
6	6	66
6	12	86

NOTE: Each Warburg flask contained 5 μmoles GSH, 6 μmoles ATP, 1 ml 0.4 *M* $NaHCO_3$, 10 μmoles glyceric acid, and 150 μg of partially purified enzyme.

glycerate phosphorylated; for example equimolar amounts of ATP and ADP decrease the activity by about one-third. The addition of Mg^{++} overcomes much of the inhibition. The conditions of these experiments were such that 100% relative activity indicated that about 30% of the glyceric acid was phosphorylated.

3-PGA is more effective in inhibiting the enzyme than is ADP. Table III shows that 6 μmoles gives complete inhibition; this is equivalent to 3 μmoles of

TABLE III

The effect of 3-PGA on glyceric acid kinase activity

3-PGA added, μmoles	Relative activity, %
0	100
2	99
4	60
6	0
8	0
10	0

NOTE: Each Warburg flask contained 5 μmoles GSH, 12 μmoles ATP, 12 μmoles Mg^{++}, 1 ml 0.4 *M* $NaHCO_3$, 10 μmoles glyceric acid, and 120 μg partially purified enzyme. The 3-PGA was a DL-mixture.

the D-isomer or the equivalent of 60% phosphorylation for the conditions employed in these experiments.

Effect of SH Inhibitors on Glyceric Acid Kinase

Glutathione and cysteine enhanced the enzyme reaction; this observation suggested that the kinase is dependent on SH groups for its activity. An experiment designed to test whether this was the case was done and the results are recorded in Table IV. *p*-Chloromercuribenzoate is the most effective

TABLE IV

The effect of sulphydryl masking compounds on glyceric acid kinase activity

Compound*	Concentration, μmoles	Relative activity, %	
		−Glutathione	+Glutathione†
None	—	100	100
p-Chloromercuribenzoate	1.1×10^{-2}	11	104
N-Ethylmaleimide	6.1×10^{-2}	35	104
o-Iodosobenzoate	3.7×10^{-2}	39	98
Iodoacetate	1.8	34	96

NOTE: The conditions of the experiment were as described in the text except that one set contained no GSH and had the various compounds in the concentrations indicated. Enzyme solutions in each flask were equivalent to 150 μg of protein.

*The solutions were adjusted to approximately pH 7.

†5 μmoles of GSH was added to each Warburg flask.

inhibitor followed by *o*-iodosobenzoate and N-ethylmaleimide. Iodoacetate also inhibits the enzyme but at 30 to 100 times higher concentrations; this is not surprising as this compound is less reactive. Five micromoles of GSH is sufficient to give complete protection against these SH-group reagents. No attempt was made to establish the lower limits of protection afforded by GSH and also no information was obtained as to whether the inhibitory effect was reversible.

Effect of Cations

Glyceric acid kinase is similar to other kinases in that it requires metal ions for its activation. Many kinases, e.g. hexokinase, require Mg^{++} or Mn^{++} for

complete activation of the enzyme. In many cases Mn^{++} can be substituted for Mg^{++} but choline kinase is completely inhibited by Mn^{++} (9). This was not found to be the case for glyceric acid kinase (Table V), in which Mn^{++} was

TABLE V

Effect of cations on the glyceric acid kinase activity

Cation added	Relative activity, %
None	14
Mg^{++}	100
Mn^{++}	80
Co^{++}	80
Ni^{++}	31
Zn^{++}	19
K^{+}	18
Fe^{++}	13
Ca^{++}	6

NOTE: The assay was carried out as described in the text except that various cations were substituted at concentrations of 12 μmoles. The assay containing Mg^{++} was assumed to give maximum activity and was designated as 100%.

almost as effective as Mg^{++}. Cobalt was as effective as Mn^{++} while both Ni^{++} and Zn^{++} were ineffective. Both K^{+} and Fe^{++} were also ineffective whereas Ca^{++} caused an inhibition of the reaction. From this study one would conclude that the divalent cation specificity for this enzyme is not as great as for some other kinases.

Substrate Specificity

A substrate specificity study in this investigation can only be a comparative one, since the enzyme preparations are not pure, If, however, there is a decrease in activity between purified and partially purified fractions for certain substrates, it does indicate that certain enzymes are being removed. The purified preparation appears to contain kinases that phosphorylate choline, glucose, and mannose (Table VI). There is no doubt that these are different enzymes as the ratios are quite different from that found for the same compounds in the partially purified preparations. The kinase that phosphorylates choline was almost completely removed by this fractionation technique. The partially purified material appears to have the ability to phosphorylate fructose and ribose and to a lesser extent mannitol and raffinose. Neither preparation phosphorylated serine, glycerol, or ethanolamine.

Isolation and Identification of Reaction Products

One of the products, 3-PGA, was isolated from the reaction mixture and subsequently identified. The following mixture was incubated at 30° C for 6 hours: glyceric acid 1 mmole, ATP and Mg^{++} 1.2 mmoles of each, GSH 0.5 mmole, 10 ml of 0.4 *M* $NaHCO_3$, and 4 ml of purified enzyme; the final volume was 100 ml. After incubation the reaction mixture was concentrated to 45 ml *in vacuo*, then deproteinized with 2 ml of 100% (w/v) TCA and heated for 10 minutes. After centrifugation the supernatant was further reduced in volume and then treated batchwise with Darco-G-60 (1 g) to remove the

TABLE VI

A study of substrate specificity on two fractions

Compound	Relative activity, %	
	Partially purified enzyme	Purified enzyme
Glyceric acid	100	100
Serine	0	0
Glycerol	—	0
Choline	74	9
Ethanolamine	0	0
Glucose	33	15
Mannose	26	16
Fructose	25	0
Mannitol	7	0
Ribose	15	0
Raffinose	3	0

NOTE: The assay was carried out as described in the text except for the above compounds which were substituted for glyceric acid at the same concentration (10 μmoles). The partially purified preparation was an ammonium sulphate fraction referred to in Table I. The activity of the two preparations was similar when tested against glyceric acid, i.e. 21.9 μl CO_2 released per hour for the purified sample and 23.1 μl CO_2 released per hour for the partially purified sample.

nucleotides. The solution was again concentrated and the acid solution was adjusted to pH 8.2 with $Ba(OH)_2$. The 3-PGA was recovered as the barium salt. The precipitate was then suspended in distilled water and brought into solution by the addition of 1 *N* HCl. The solution was once again treated with Darco-G-60 and then precipitated as the barium salt. The salt was then washed and dried.

The crude barium salt was further purified on a Dowex-1-formate column (15). The salt was dissolved in a small quantity of 0.05 *N* HCl and the barium was removed on a small column of Amberlite IR-120. The free 3-PGA was then added to a Dowex-1-formate column (height 20 cm, diameter 2 cm). It was first washed with 200 ml of water which eluted any free glyceric acid; the column was then treated with 600 ml of 4 *N* formic acid and finally with 500 ml of a mixture containing 4 *N* formic acid + 0.2 *M* ammonium formate. Ten-milliliter samples were collected and the 3-PGA appeared in the last few tubes. After concentration and removal of the formate the 3-PGA was identified by paper chromatography by comparing with an authentic sample. The sample was run in two different solvents: (*a*) methanol, ammonium hydroxide, water (6:1:3) or (*b*) ethyl acetate, acetic acid, water (3:3:1). The R_f of the enzymatically produced 3-PGA agreed with the authentic compound, thus giving good evidence that 3-PGA had been formed.

Discussion

The properties for glyceric acid kinase described in this paper are similar to those reported earlier for mammalian tissue (5, 6). The plant enzyme like the rat liver enzyme (6) is rather unstable during purification and thus presents difficulties in handling. The yields and purification for both were low. The pH optimum was similar to the enzyme found in horse liver (pH 7.4 to 7.7

(5)) and rat liver (pH 7.2 to 7.4 (6)). All three enzyme preparations require Mg^{++} as a cofactor and the Michaelis constants are of the same order.

The direct phosphorylation of glyceric acid is another pathway for deriving this compound in plants. However, it is not the only pathway, as spinach extracts synthesize this material from a condensation of a pentose and CO_2 (16). In acetone powders of sugar beet leaves there is still another pathway; the oxidation of glyceraldehyde-3-phosphate by TPN to 3-PGA (17). It would therefore appear that this compound can be derived by a number of different pathways.

What is the significance of 3-PGA in the tissues of mammals and plants? Undoubtedly it serves as an intermediate step to many compounds of which only a few will be mentioned here. This phospho-compound apparently gives rise to phosphopyruvic acid, which has been found in both fish muscle (18) and in human erythrocytes (19). This acid in turn probably is dephosphorylated and from there finds its way into the general metabolic pathways of pyruvate. It appears that 3-PGA is a precursor for both phosphoserine and serine. The formation of phosphoserine from 3-PGA has been demonstrated in higher plants by Hanford and Davies (8). They suggest the same mechanism as that indicated earlier by Greenberg and co-workers (7). There appears to be no evidence that serine is phosphorylated enzymatically by ATP (20). Unpublished work in this laboratory supports this view. Therefore if this is true then the above pathway to phosphoserine has very significant implications, particularly in the biosynthesis of phosphoproteins.

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MITOTIC RATE IN THE EPIDERMIS OF WARM- AND COLD-ACCLIMATED RATS[1]

O. HÉROUX

With the technical assistance of DONNA WRIGHT

Abstract

The effect of the environmental temperature on the mitotic activity in the skin of warm- and cold-acclimated rats was estimated with the colchicine method. On rats kept at 30° C for 3 months, the skin temperature was found to vary from 30.4° C on the ear, to 35.3° C on the back. Least-square linear relationships were established between log induction rate (rate at which cells start division), log mitotic duration, and tissue temperature. The colder the epithelium, the lower was the induction rate and the longer was the mitotic duration. Depending on their temperature, the different epithelia studied could be classified as far as their mitotic activity was concerned, in the following decreasing order: duodenum, back, tail, foot, and ear. For a 7° C difference in temperature between duodenum and ear, there was a sevenfold difference in mitotic duration, which reveals an unusual Q_{10} of 10.

The data obtained on similar epithelial tissues of cold-acclimated rats supplied some evidence of adaptation in the mitotic rate of the back skin. In the ear, foot, and tail skin the colchicine method failed to give quantitative data because mitosis was proceeding too slowly. It even failed in rats maintained in the cold for 118 days. When this finding is considered together with the observations reported previously that after 3 weeks of cold exposure, mitotic activity in the ear is resumed, after having been stopped almost completely, it appears that the adaptation that has taken place in this cold tissue is one that allows the cells to overcome the mitotic blocking effect of cold but not one which allows mitosis to proceed at a much faster rate than would be indicated by the Q_{10} observed on the epithelia of the "30° C rats".

Introduction

Although homeothermic animals maintain a constant central body temperature, their peripheral tissues can cool to an extent which is comparable to that seen in cold-blooded animals. This has been found by Irving and Hart (1) to occur to a remarkable degree in seals which maintain skin temperatures close to 0° C in sea water during the winter, while maintaining a central temperature of 37° C. In white rats kept at 6° C for 4 months, skin temperatures of 9 to 11° C have been found on the tail and feet (2).

Such a lowering of tissue temperature greatly reduces cellular activity. It has been reported, for example, that hair, beard, and nails grew at a slower rate on men living in the Arctic (3) or in the Antarctic (4) than in those living in temperate climates. Storey and Leblond (5) found that the renewal of the foot epidermis was less rapid in rats kept for 7 hours on a cage floor maintained at 10 to 12° C than in rats kept on a cage floor maintained at 25 to 35° C.

A recent histological study on non-freezing cold injury (6) revealed that in the ears of white rats continuously kept at 6° C the mitotic activity in the epidermis was almost completely blocked during the first 21 days. A similar phenomenon has been observed in the newt exposed to 3° C (7) and in tissue

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cultures kept at 16° C (8, 9). However, in the case of the rats' ears (6), mitotic activity was resumed after 3 weeks although the rats were still in the cold. This recovery was a new phenomenon that, to our knowledge, had never before been reported. The increase in number of mitotic cells was further studied and a direct measurement of the rate at which epidermal cells divide before and after acclimation was attempted by the colchicine method described by Storey and Leblond (5).

Experimental Procedures

Male Sprague–Dawley rats of an average body weight of 215 g were randomly assigned to groups which were kept for different lengths of time in constant-temperature rooms maintained at 30° C or 6° C. Each rat was kept individually in a wire cage and had an ad libitum supply of Master Fox Chow and tap water. In each room, the artificial light was on from 8 a.m. to 8 p.m. After exposure, the rats were killed by excess of ether. At the same time, the others were injected either intraperitoneally or subcutaneously with a dose of colchicine (see Table I) and killed either 3, 6, 12, or 24 hours later. The rats that were killed after 12 or 24 hours received an additional dose of colchicine every 6 hours.

TABLE I

Effect of different doses of colchicine on mitosis of ear epidermis after acclimation to 30° C or 6° C

Acclimation group	Route of injection	Dose, mg/100 g	Time after colchicine, hours	No. of mitotic cells per 2000 cells			
				Prophase	Metaphase	Anaphase	Telophase
30° C	Control	—	—	21	18	2	28
	Intraperitoneal	0.1	6	18	34	0	0
	Subcutaneous	0.1	6	23	57	0	0
		0.25	6	23	51	0	0
6° C	Control	—	—	25	37	3	29
	Intraperitoneal	0.1	6	62	23	3	9
	Subcutaneous	0.1	6	53	18	0	22
		0.1	12	62	41	0	13
		0.1	24	24	50	0	12
		0.25	6	35	20	0	8
		0.5	6	67	22	0	21

NOTE: Each experimental group contained four rats and underwent an acclimation period of 28 days.

After death, a 3-mm × 10-mm portion of skin involving the epidermis and subjacent tissues was taken from the ear, tail, foot (plantar surface), and the back. A cross section of the duodenum was also taken. All tissues were dropped immediately in Bouin fixative and 24 hours later, they were dehydrated in alcohol, cleared in toluol, infiltrated, and embedded in paraffin (Fisher Tissuemat, melting point 54–56° C), cut on the microtome at 10 μ, and stained with haematoxylin and eosin.

With the aid of an eyepiece micrometer, 2000 nuclei in the Malpighian layers of the epithelium were counted under oil immersion on one section of each tissue for each rat. Cells were counted only in the flat portions of the epidermis and not in the root sheaths of the hair. Among these 2000 cells, the different stages of mitosis were identified by the method described by Leblond and Stevens (10) and then counted separately.

The mitotic counts, obtained on the acclimated rats which were not treated with colchicine, were considered to be the "initial" counts in the calculations for mitotic duration, which were carried out as follows. Assuming that the drug blocks the metaphase process, and knowing that the initial and post-colchicine counts were separated by a time interval "t" long enough for existing metaphases, anaphases, and telophases to go to completion, then the rate of induction of mitosis is M_2/t, and the duration of mitosis is the total initial count divided by the rate, i.e.

$$d = \frac{P_1 + M_1 + A_1 + T_1}{M_2} t,$$

where P_1, M_1, A_1, T_1 are the initial numbers of prophases, metaphases, anaphases, and telophases respectively, and M_2 is the number of metaphases after colchicine injection. The formula implies that at time t all the initial metaphases would have completed division and reached the resting stage, so that all metaphases counted at time t after colchicine injection consist of mitoses started during that time.

With a fine thermocouple attached to the tip of a wooden stick, the surface skin temperature was recorded on the tail (1 inch from the base), the back (on the midline), and the plantar surface of the foot of unanesthetized rats resting in their cages at their own acclimation temperature. The duodenal temperature was assumed to be the same as the rectal temperature, which was measured with a thermocouple inserted to a depth of 6 to 7 cm. Accurate measurements of the ear temperature on undisturbed animals left in their cages could not be obtained, but according to Leblanc (11), the surface ear temperature of rats acclimated to 6° C is on the average 18° C. For the 30° C rats an average ear temperature of 30.4° C was arbitrarily chosen since it was presumably higher than environmental temperature (30° C) and lower than temperatures of the less exposed tail and foot (31.1° and 30.7° C respectively).

Results

Mitotic Duration in "30° C Rats"

Intraperitoneal or subcutaneous injections of 0.1 mg/100 g body weight of colchicine arrested mitosis at the metaphase stage in all the tissues studied (ear, tail, foot, back, and duodenum) of normal rats acclimated to 30° C for 28 days (Tables I and III, A). Six hours after the injection, the number of metaphases had increased and there were no longer any cells at the anaphase or telophase stages. From the increase in the number of metaphases, the induction rate was found to be greater in the warm tissues than in the cold ones. While only 9.5 new cells per 2000 cells appeared every hour in the ear epidermis, there were about 18 new ones appearing every hour in the tail or the foot, 41.6 in the back skin, and 206.6 in the duodenum. Correspondingly the mitotic duration was calculated to be 7.3, 5.7, 4.7, 2.4, and 0.98 hours for the ear, tail, foot, back, and duodenum respectively.

Mitotic Duration in "6° C Rats"

In the "6° C acclimated rats", 3 or 6 hours after colchicine injection, there was an accumulation of cells at the metaphase stage and an almost complete

TABLE II

Average of transformed cell counts in the ear of animals maintained at 6° C (log of counts +1)

Treatment	Time after colchicine injection (hours)	Metaphases	Telophases
Normal	0	1.478±0.033†(5)	1.430±0.099 (6)
Colchicine	24	1.655±0.036 (4)	1.116±0.033 (5)
Difference (N−C)		+0.177±0.049**	−0.314±0.104*

NOTE: Number of rats per group given in parentheses.
*Difference statistically significant at the 5% level.
**Difference statistically significant at the 1% level.
†Standard error.

disappearance of telophases in the duodenum and back epidermis but not in the ear, tail, or plantar epidermis (Table III, B). In the ears, no apparent blocking of mitosis appeared 6 or even 12 hours after the intraperitoneal or subcutaneous injections of 0.1, 0.25, or even 0.5 mg/100 g of colchicine (Table I). The number of metaphases did not increase and telophases did not disappear.

When animals were injected every 6 hours, with 0.1 mg of colchicine, 24 hours after the first injection there was a substantial increase in the number of blocked metaphases and a 50% reduction in the number of telophases. Both differences were statistically significant (Table II). The animals, however, were all in a very poor condition: they were hypothermic and suffered from diarrhea. The presence of telophases, even after 24 hours of colchicine treatment, could be due to either one of two causes: (*a*) an incomplete blocking of mitosis or (*b*), a more likely possibility, the mitotic process was so slow that the telophases were cells already in division at the beginning of the experiment which did not have time to complete mitosis.

The back epidermis and duodenal epithelium lend themselves readily to the calculation of the induction rates, which were found to be 38 and 202.3 dividing cells per 2000 cells per hour, and of the mitotic durations, which were 2.7 and 1.17 hours respectively. At those two locations, the mitotic activity appeared to be essentially the same in the "30° C and 6° C acclimated rats".

By plotting against tissue temperature the log induction rate and the log mitotic duration of all the tissues of the "30° C" rats and those for the back and duodenum of the "6° C" rats, linear relations were revealed (Fig. 1), which were expressed by the following least-square equations:

$$\log \text{ induction rate} = -3.617+0.1562 \text{ (tissue temperature)},$$
$$\log \text{ mitotic duration} = 3.839-0.1010 \text{ (tissue temperature)}.$$

Assuming that after 4 weeks of cold exposure the mitotic activity in the ear, foot, and tail was not significantly altered by adaptation per se, or in other words that the linear relationship still obtained at 18° C or 11° C, a mitotic duration of 105 hours (4 days) for the ear and 487 hours (20 days) for the foot and 476 hours (15 days) for the tail were calculated. Similarly induction rates of 0.156 cell per hour per 2000 cells for the ear and 0.015 cell per hour per 2000 cells for the foot and tail were obtained by extrapolation.

TABLE III

Induction rate and mitotic duration in epidermis (and duodenum) of rats

Tissues	Tissue temp., °C	Treatment: Accl., days	Groups	Time after colchicine, hr	No. rats	No. of mitotic cells per 2000 cells: Prophase	Metaphase	Anaphase	Telophase	Mitotic index, %	Induction rate, new cells per hour per 2000 cells	Mitotic duration (hours)
A. Rats acclimated to 30° C												
Ear	30.4	28	Normal		4	21	18	2	28	3.5		
			Colchicine	6	4	23	57	0	0		9.5	7.3
Foot	30.7	28	Normal		4	30	19	5	50	5.2		
			Colchicine	6	4	30	105	0	2		17.5	5.9
Tail	31.1	28	Normal		4	42	12	2	30	4.3		
			Colchicine	3	4	20	55	0	1		18.3	4.7
Back	35.3	28	Normal		3	41	19	1	37	4.9		
			Colchicine	3	4	33	125	0	0		41.6	2.4
Duodenum	37.5	28	Normal		4	46	91	9	58	10.2		
			Colchicine	3	4	28	620	0	0		206.6	0.98
B. Rats acclimated to 6° C												
Ear	?	28	Normal		6	25	37	3	29	4.2		
	?	28	Colchicine	6	4	26	39	0	29		(0.1565)	(105.0)
	18	28	Colchicine	24	5	24	50	0	12		0.54	174.0
	?	118	Normal		4	81	28	0	32	7.0		
	?	118	Colchicine	12	4	108	10	0	35		—	—
Foot	11.4	28	Normal		4	46	10	2	20	3.9		
		28	Colchicine	6	4	49	14	1	45		(0.0146)	(487.0)
Tail	11.5	28	Normal		4	53	19	2	38	5.6		
		28	Colchicine	6	3	35	18	2	20		(0.0151)	(476.0)
Back	32.0	28	Normal		4	48	16	1	36	5.0		
			Colchicine	3	4	42	114	0	1		38	2.7
Duodenum	37.5	28	Normal		4	49	113	11	64	11.8		
		28	Colchicine	3	4	23	607	0	0		202.3	1.17

*Extrapolated values are given in parentheses.

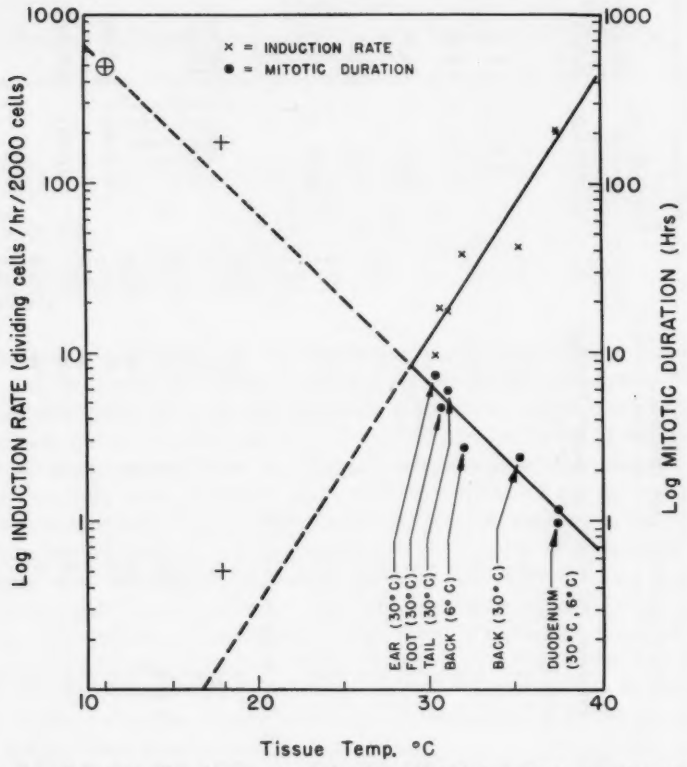

FIG. 1. Correlation lines between log induction rate (×), log mitotic duration (○), and tissue temperature, calculated by the least-square method; + represents the calculated values for the ear epidermis of 6° C rats, and ⊕ the values for the plantar epidermis.

To clarify the results, the observed induction rate as well as the extrapolated values are expressed (in rounded figures) as cells starting division in every 400,000 cells per hour and to complete the general picture, mitotic duration is also given.

Tissue	Acclimation temperature	Tissue temperature	Induction rate per hour per 400,000 cells	Mitotic duration (hours)
Duodenal epithelium	(30° C)	37.5	41,300	0.98
Back epidermis	(30° C)	35.3	8,300	2.40
Back epidermis	(6° C)	32.0	7,600	2.70
Tail epidermis	(30° C)	31.1	3,500	5.90
Ear epidermis	(30° C)	30.4	1,900	7.30
Ear epidermis	(6° C)	18.0	30	105.00
Tail epidermis	(6° C)	11.5	3	476.00

Obviously these figures are not absolute but on a relative basis they illustrate the general correspondence between mitotic activity and the tissue temperature.

Discussion

The mitotic duration measurements in the epidermis of the "30° C" rats revealed a direct relationship between the log induction rate, the log mitotic duration, and the tissue temperature with an unusual Q_{10} of 10. A similar Q_{10} was also observed by Storey and Leblond (5) when they compared the mitotic activity in the plantar skin of rats kept on cage floors maintained at different temperatures. A temperature coefficient of this magnitude suggests a cumulative inhibitory effect of the cold temperature on many processes. Whatever is the explanation for such a large temperature effect, it certainly accounts for the absence of an accumulation of metaphases in the ear, foot, and tail of the "6° C rats" 6 hours after the injection of the colchicine.

By extrapolation, it appears that in the ear and the tail of the "6° C rats", which maintained a surface ear and tail temperature of 18° C and 11° C respectively, an epidermal cell would take 4 days (105 hours) and 15 days (476 hours) to complete division instead of 7 and 6 hours as in the ear and tail of "30° C rats". The values of these extrapolations are limited by the validity of the different assumptions that were made to justify them, but the order of magnitude agrees with the observations made on the ear at 24 hours after colchicine treatment. In this case, the observed measurements were not significantly different from the extrapolated values. Considering the large experimental error in these measurements, the extrapolated mitotic duration of 4 days is of the same order as the one measured 24 hours after colchicine (7 days). Moreover, as reported in a previous paper (6) after 14 days at 6° C, the number of blocked metaphases (arrested by cold alone) increased to 94.8 per every 2000 cells which would indicate an even longer mitotic duration of 10 days. At these rates it is not surprising that 6 and 12 hours after colchicine and even after a fivefold increase in the dose, no accumulation of metaphases could be observed in the cold ear, foot, and tail. There is no doubt that colchicine was just as effective in the "6° C" rats as in the "30° C" rats since its effect was observed in the ear 24 hours after injection and since it had a normal action on the duodenum and the back skin.

Apparently, cold adaptation did not affect the mitotic activity of the duodenum of "6° C" rats; the induction rate and mitotic duration in that tissue were essentially the same in both "30° C" and "6° C" rats, a result in agreement with the failure of the environmental temperature to affect the duodenal temperature. In the slightly colder skin of the back, there is, however, some evidence for cold adaptation; essentially the same mitotic duration was observed in both groups although the "6° C" rats maintained a back surface temperature 3° C lower than in the "30° C" rats. According to the linear equation, for a temperature of 32° C the mitotic duration should have been 4.05 hours instead of the observed 2.7 hours.

In rats exposed to cold for 118 days, the mitotic activity in the ears was still very slow since 12 hours after colchicine injection there was still no accumulation of cells at the metaphase stage (Table III, B). When this finding is considered together with those reported previously (6) that, after 3 weeks, mitotic activity

is resumed in the cold ear, it appears that adaptation that has taken place is one that allows the cells to overcome the mitotic blocking effect of cold but is probably not one which allows mitosis to proceed at a much faster rate than the Q_{10} observed on the epithelia of the "30° C" rats would indicate. If under those conditions, the skin tends to return to a normal thickness (6) it must mean that a new equilibrium between the rate at which new cells are produced and the rate at which they desquamate is setting in.

If these results can be extended to other species, they indicate that in skins maintained at 0° C, such as those in the Arctic seals (1), the renewal of the epidermal cells must be almost nil, unless through some adaptation cellular division can take place at much lower temperatures.

Acknowledgment

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BIOSYNTHESIS OF THE COUMARINS. TRACER STUDIES ON COUMARIN FORMATION IN HIEROCHLOË ODORATA AND MELILOTUS OFFICINALIS[1]

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Abstract

Coumarin formation has been studied with C^{14} in the perennial grass, *Hierochloë odorata*, and in yellow sweet clover, *Melilotus officinalis*. In general the latter species yielded inconsistent data. In *Hierochloë*, *o*-coumaric, cinnamic, and shikimic acids and L-phenylalanine were the best of 10 compounds tested as coumarin precursors, the first two at least being incorporated with little randomization of C^{14}. Acetate was more poorly utilized. It was concluded that the aromatic ring of coumarin arises via the shikimic acid pathway in preference to acetate condensation. When the time of metabolism was varied, *o*-coumaryl glucoside and free *o*-coumaric acid rapidly acquired high specific activities from cinnamic acid-C^{14}, but coumarin and melilotic acid became active much more slowly. A lag in the acquisition of C^{14} by coumarin for the first 6 to 8 hours was followed by a rectilinear increase until at least 24 hours. Much the greatest accumulation of C^{14} was found in *o*-coumaryl glucoside during this entire period. Furthermore, this compound when fed to *Hierochloë* is comparable to cinnamic acid as a coumarin precursor. These findings suggest a possible function for *o*-coumaryl glucoside or a derivative in coumarin biosynthesis.

Introduction

Despite the large number of investigations in recent years into the formation of phenolic compounds in plants (cf. 1) the problem of the origin of the coumarins has attracted relatively little attention. This is perhaps surprising in view of the widespread distribution and variety of structure associated with this class of compound (2). There has been, however, some speculation about possible biosynthetic pathways. The earlier theories have been critically reviewed by Reppel (3). The more recent hypotheses (4, 5), developed since the mechanism of aromatization has become better understood, have been alike in the assumption that coumarins are derived from phenylpropanoid acids of the cinnamic acid type. Haworth (6) some years ago proposed an ingenious scheme to explain the formation of 7-hydroxycoumarins. Geissman (5) has suggested that coumarin formation may involve a phenylpropanoid β-keto acid, and that such an intermediate may arise by a $C_6,C_1 + C_2$ condensation, which would be analogous to that already established for the synthesis of aliphatic fatty acids.

Experimental work on the biochemistry of the formation of coumarins appears to have been limited almost entirely to scopoletin (I*c*) and coumarin itself (I*a*). Reznik and Urban (7) fed ferulic acid-3-C^{14} to leaves and young plants of *Helianthus annuus*, *Triticum vulgare*, and *Zea mays* by vacuum

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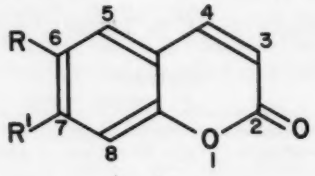
infiltration, and after allowing 3 days' metabolism, isolated radioactive scopoletin, but no indication of the efficiency of ferulic acid as a precursor was given.



I

(*a*) R = R′ = H
(*b*) R = H; R′ = OH
(*c*) R = OCH_3; R′ = OH

Reid (8) has made quantitative comparisons of carbon dioxide and three organic compounds labelled with C^{14} as scopoletin precursors in *Nicotiana tabacum*. He found that the carbon-14 of L-phenylalanine was incorporated into scopoletin with about one-fifth the dilution of carbon dioxide, whereas that of acetic and phenylacetic acids underwent appreciably greater dilution than carbon dioxide. However, the dilution even in the case of phenylalanine was relatively high.

Little is known of the sites at which coumarins are formed. Mothes and Kala (9) have shown with the aid of tissue cultures that formation of scopoletin and umbelliferone (I*b*) can take place in the roots of *Atropa belladonna* grown on a medium containing carbohydrate as the carbon source. As these authors themselves pointed out, their experiments gave no indication that the roots are the preferred synthetic site and by no means excluded the possibility of formation in other organs.

Very recently the problem of the biosynthesis of coumarin itself has been studied by Kosuge and Conn (10) using *Melilotus alba*. C^{14}-Labelled acetate was not measurably incorporated into coumarin, and glucose, DL-phenylalanine, and cinnamic acid were increasingly effective as coumarin precursors. They, too, were unable to obtain efficient conversion of any precursor to coumarin, and even the carbon-14 of cinnamic acid underwent a dilution of 7500 during the reactions involved. Further experiments on the fate of labelled coumarin administered to *Melilotus* led them to conclude that this compound is rapidly metabolized to melilotic acid and its glucoside and to a lesser extent to *o*-coumaryl glucoside. They inferred that, because of this rapid metabolism, it was difficult to obtain high levels of carbon-14 in coumarin by the administration of labelled precursors to this species.

In most of the studies to be reported here, the experimental plant has been the perennial grass, *Hierochloë odorata*, which has a coumarin content comparable to that of *M. alba* (11). Because of the ease with which this grass can be cultivated the year round under indoor conditions, and the consistency with which it synthesizes large amounts of coumarin once established, it has proved to be an excellent subject for the investigation of coumarin biosynthesis. Using

it we have been able to demonstrate much higher conversion efficiencies of several precursors to coumarin than any yet published for analogous reactions.

Experimental

All melting points are corrected.

Cultivation of the Plants

Sweet grass (*Hierochloë odorata* Beauv.) was collected from the wild state near Saskatoon and transplanted to coarse quartz sand which was irrigated at intervals from below with a nutrient medium (12). Early experiments were conducted on greenhouse-grown plants maintained at temperatures of 25.5° during the day and 17° at night. As these conditions did not appear suitable for rapid coumarin synthesis the plants were transferred to artificial growth chambers where they were exposed to illumination from "cool white" fluorescent tubes and incandescent bulbs at a total intensity of ca. 13,000 lumens/m^2 (1200 ft-c) with a day length of 18 hours. The temperature range was about 20–23°. These conditions proved entirely satisfactory.

Yellow sweet clover (*Melilotus officinalis* Lam. var. Erector) could not be grown satisfactorily in the growth chamber under the above conditions, as the long day induced premature flowering. Experiments on this species were conducted either on soil-cultivated plants from out-of-doors or on greenhouse-grown plants from gravel culture (12).

Synthesis of C^{14}-Labelled Compounds

Randomly labelled L-phenylalanine and sodium acetate labelled in carbons-1 and -2, respectively, were purchased from Atomic Energy of Canada, Ltd., Ottawa. Syntheses of shikimic acid-R-C^{14} (13) and cinnamic acid-3-C^{14} (14) have been described in earlier papers. The samples of cinnamic acid-1-C^{14} and ferulic acid-2-C^{14} synthesized from appropriately labelled malonic acid under the conditions previously described (14) were obtained through the courtesy of Dr. A. C. Neish.

Coumarin-2-C^{14}.—Diethylmalonate-1,3-C^{14} (267 mg, purchased from Atomic Energy of Canada Ltd.) was reacted with salicylaldehyde (240 mg) under the conditions described by Horning, Horning, and Dimmig (15). The crude 3-carbethoxycoumarin (290 mg) from the reaction was refluxed with 0.4 g of sodium metabisulphite in 1.5 ml of water for 30 minutes, 1.5 ml of concentrated sulphuric acid was added, and the heating was continued 1 hour (16). The mixture was poured into water, and extraction with ether yielded coumarin-2-C^{14} which, after sublimation (55°, 2 mm), weighed 190 mg, m.p. 68° (78% based on diethyl malonate). The radioactive carbon dioxide released in the decarboxylation was passed through a potassium permanganate solution and collected in an alkali trap.

o-Coumaric acid-1-C^{14}.—Coumarin-2-C^{14} (144 mg) was refluxed for 2 hours with 2 ml of 10% sodium hydroxide solution and 9 mg of yellow mercuric oxide (17).* Mercuric sulphide was precipitated by passage of a slow stream of hydrogen sulphide for a few minutes, followed by acidification, and the mixture

*The conditions of Seshadri and Rao (27), whose paper was unknown to us at this time, are preferable for converting coumarin to *o*-coumaric acid (*vide infra*).

was then extracted with ethyl acetate. The crude product from the extract was decolorized with charcoal and recrystallized from hot water, yielding 100 mg of *o*-coumaric acid-1-C^{14}, m.p. 215° d. (62% based on coumarin).

Melilotic acid-1-C^{14}.—*o*-Coumaric acid-1-C^{14} (104 mg) dissolved in 30 ml of water was neutralized to pH 7 with sodium bicarbonate. Reduction was carried out for 2 hours under 2.7 atmospheres of hydrogen in the presence of 75 mg of palladium–charcoal catalyst (5% Pd). The product, melilotic acid-1-C^{14}, after crystallization from benzene–petroleum, weighed 65 mg, m.p. 87° (62% based on *o*-coumaric acid). On the somewhat larger scale employed in preliminary runs with non-radioactive material, nearly quantitative yields of *o*-coumaric and melilotic acids were obtained.

Salicylic acid-$C^{14}OOH$.—*o*-Bromophenylbenzyl ether, (1.58 g, 6 mmoles) prepared from *o*-bromophenol and benzyl chloride (cf. 18), in 20 ml of dry ether, was cooled in liquid nitrogen and the tube evacuated. A solution of *n*-butyl lithium in pentane (19) (4.6 ml, 1.08 *N*) was added through a serum bottle cap with a syringe, and the mixture shaken at −20° for 10 minutes. The flask was reconnected to the vacuum line, immersed in liquid nitrogen, and carbon dioxide generated from 885 mg of barium carbonate-C^{14} was allowed to condense. The flask was again shaken at −20° for 15 minutes and the product was then decomposed with 5 ml of 6 *N* hydrochloric acid. Alkaline extraction of the ether layer yielded 0.51 g of salicylic acid benzyl ether. (Yields of 0.7 g are obtainable.) This was reduced in 30 ml of methanol under 2 atmospheres of hydrogen for 2.5 hours in the presence of 0.2 g of palladium–charcoal catalyst at room temperature. After charcoal decolorization of the crude product (275 mg) and recrystallization from hot water, salicylic acid-$C^{14}OOH$, 200 mg, m.p. 158° (32% based on barium carbonate), was obtained.

o-Tyrosine (*o-hydroxy-β-phenylalanine*).—Salicylic acid-$C^{14}OOH$ (490 mg) was refluxed 2 hours with acetic anhydride (0.4 ml) in benzene (3.5 ml). The product obtained upon removal of the solvent was treated with thionyl chloride (1 ml) and benzene (2 ml) at 80° for 5 hours, yielding acetylsalicyloyl chloride.* After distillation at 140 to 150° (bath)/12 mm, the acid chloride was subjected to a Rosenmund reduction as described by Brown and Neish (20), except that Teflon sleeves instead of grease were used on the glass joint. After evolution of 90% of the calculated hydrogen chloride the xylene solution was filtered and the filtrate stirred for 2 hours with 7 ml of *N* sodium hydroxide. From this reaction mixture was isolated, by distillation at 12 mm, 214 mg of salicylaldehyde-$C^{14}HO$.

The salicylaldehyde was dissolved in glacial acetic acid (2.3 ml) plus one drop of piperidine and to this was added 2-mercaptothiazol-5-one (228 mg) (21). The mixture was warmed until solution was complete and then allowed to stand 12 hours. Addition of water resulted in the eventual separation of 201 mg of 4-(2′-hydroxybenzylidene)-2-mercaptothiazol-5-one. This was refluxed 2 hours with red phosphorus (0.24 g), acetic acid (1.2 ml), and hydriodic acid (0.5 ml, sp. gr. 1.5). After removal of the phosphorus as much hydrogen iodide as

*A more direct route involving the preparation of salicylaldehyde benzyl ether from the corresponding acid and its condensation with the thiazole had to be abandoned because the acid chloride necessary for the Rosenmund reduction could not be obtained pure.

possible was removed by repeated addition of water followed by vacuum distillation. The residue was then dissolved in 2 to 3 ml of water, the solution decolorized with charcoal, and ammonia added to effect separation of the amino acid. The first crop of crystals, after recrystallization, yielded 27 mg of *o*-tyrosine-3-C^{14}, m.p. 250°. Additional quantities of less active *o*-tyrosine were obtained by the addition of non-radioactive carrier to the combined mother liquors. Yields up to 80% are obtainable in the reductive hydrolysis of the thiazolone.

Administration of Labelled Compounds

The age and size of the *Hierochloë* plants grown in artificial growth chambers did not appear to be a critical factor in their ability to synthesize coumarin. Usually shoots of 30 cm or more in length were used; when growth had reached this stage an odor of coumarin was noticeable in the vicinity of the plants. The grass was cut near the base with scissors and subsequently the ends were trimmed under water with a razor blade and immersed in aqueous solutions of the compounds being fed (4 ml of solution, ca. 0.025 *M*, per 25 g fresh weight). Water-insoluble acids were dissolved in a calculated volume of 5% sodium bicarbonate and fed as sodium salts, pH 6 to 7. A temperature of 18 to 20° was maintained during administration of the labelled compounds and subsequent metabolism, which took place under artificial or natural illumination in excess of 5000 lumens/m^2 (500 ft-c). Absorption of the solution required 1.5 to 2 hours; the time when the solution had been completely absorbed was noted and recorded as zero hours.

After harvesting, plant material was worked up at once if practical, or otherwise frozen quickly and stored in the frozen state until used.

Isolation of Coumarin

In the early stages of this work coumarin was isolated by a procedure based on extraction of the plant material with hot 80% ethanol. This procedure was abandoned because of difficulty encountered in purifying the product, and will not be described in detail. The method of Duncan and Dustman (22) involving successive steam distillations under partial vacuum was adopted instead. Before steam distillation the plant material was disintegrated with a convenient volume of hot water in a VirTis homogenizer or a Waring blendor. The steam distillate was passed successively through columns of Amberlite IR-4B(OH^-) and IR-120(H^+) ion exchange resins, and extracted several times with ether. The slightly yellow residue of crude coumarin left on removal of the ether was sublimed at 50 to 55°, 1 mm. The purified coumarin, melted at 70 to 70.5°, was homogeneous when chromatographed in the vapor phase, and paper chromatography revealed the presence of no phenolic contaminants. The yield usually approximated 2 mg per gram fresh weight of plants.

Isolation of Phenolic Acids and o-Coumaryl Glucoside

To the residue from the steam distillation was added a convenient volume of absolute ethanol (ca. 300 ml) and the mixture was heated to 70–75° in a water bath. Concentrated hydrochloric acid (1 ml) was stirred in to ensure an acid reaction for extraction of carboxylic acids, and the mixture was filtered through

a medium-porosity glass frit. The filtrate was concentrated to a syrup *in vacuo*. The residue from the evaporation was taken up in two 20-ml portions of boiling water and the solutions were filtered with the aid of diatomaceous earth. The combined filtrates were concentrated to about 15 ml in an air jet on a steam bath, the pH was adjusted to below 2 if necessary, and the mixture was extracted continuously with ether for at least 12 hours. During this time crystals accumulated on the walls of the receiver, and showed no further increase in amount after more prolonged extraction.

The pale yellow extract was decanted free of the crystals and shaken with 5% sodium bicarbonate solution. The residual ether contained some coumarin which could be recovered by sublimation if the total carbon-14 content of this compound was to be calculated. Acidification of the aqueous phase and continuous back extraction for 2 to 3 hours with ether yielded an acid fraction which was chromatographed on a 46×57 cm sheet of Whatman No. 3MM paper in 1% acetic acid, using the ascending technique. By examination of the chromatogram under long-wave ultraviolet light (366 mμ) and spraying of test strips with diazotized *p*-nitroaniline reagent (23) bands of several phenylpropanoid phenolic acids were readily located. These were identified by comparison with authentic samples; in order of increasing R_f they were ferulic, *p*-coumaric, *o*-coumaric, and melilotic acids. The first three were well separated from each other and from other phenolic compounds, of which several others were present in small amounts. The melilotic acid was associated with several phenolic contaminants, and it was necessary to rechromatograph it in toluene – acetic acid – water (4:1:5) by the descending technique (24) to eliminate these. In each case the bands were extracted about 1 hour continuously with hot methanol and the extracts blown to dryness. The specific radioactivity of each sample was determined essentially by the method of McCalla and Neish (25). After the addition of inactive carrier, melilotic acid was recrystallized from benzene and the other acids from water.

The crystals which separated during the ether extraction were dissolved in hot water, the solution was decolorized with charcoal (Darco G-60), and the material recrystallized from hot water, yielding a virtually colorless product melting with decomposition at about 240°. It was readily hydrolyzed with β-glucosidase to give *o*-coumaric acid and glucose. A comparison of its X-ray diffraction pattern with that of a synthetic sample of *o*-coumaryl glucoside showed the two substances to be identical. In the early experiments the glucoside was hydrolyzed with β-glucosidase and the resulting *o*-coumaric acid was purified by recrystallization. Later this was found to be unnecessary, as additional recrystallizations of the glucoside were shown not to alter the specific activity, and the glucoside was submitted for C^{14} analysis as such.

Degradation of Coumarin

Carbon-4 of coumarin (cf. I) was recovered by the following procedure (26): To the coumarin sample (50 mg or more) in a nickel crucible was added four parts of sodium hydroxide pellets and 1 to 2 ml of water. The crucible was warmed gently with agitation until all the coumarin had dissolved, and most of

the water was then removed under an air jet on the steam bath. The crucible was carefully lowered into a Wood's metal bath at 240 to 250° and the contents were stirred until reduced to a dry powder. Heating was maintained for 2 hours with occasional mixing, during which time the yellow mass became almost white. Water was added, and acidification of the solution yielded crude salicylic acid, which was collected by filtration. This product was dissolved on the frit with 5% sodium bicarbonate solution, the solution extracted with ether to eliminate any residual coumarin, and the salicylic acid again recovered by acidification. It was decolorized with charcoal and recrystallized once from water. An additional recrystallization did not significantly alter the specific activity.

Carbon-2 of coumarin was recovered as follows: A solution of coumarin in cold sodium hydroxide was shaken for 0.5 hour with yellow mercuric oxide (27), giving almost quantitative conversion to *o*-coumaric acid. The product was purified by solution in 5% sodium bicarbonate, extraction of this solution with ether, recovery of the *o*-coumaric acid by acidification, recrystallization from hot water, and finally sublimation. It was then decarboxylated thermally. The reaction vessel was a small pear-shaped flask through which carbon-dioxide-free nitrogen could be passed. The effluent gas was passed through a scrubber containing *N* sulphuric acid and then into a trap containing carbonate-free 0.2 *N* sodium hydroxide. Some caution was necessary during the heating because of the tendency for *o*-coumaric acid to sublime at the decarboxylation temperature. The flask containing the acid (in solid form) was partially immersed in the metal bath at 190–195° and a slow stream of nitrogen passed through. The temperature was slowly raised to 205° and maintained there until no acid was visible in the bottom of the flask (some sublimes on the upper walls). The metal bath was then raised to the neck of the reaction flask, a stopcock on the nitrogen inlet closed, and the temperature raised rapidly to 210°. Finally, sweeping with nitrogen was resumed for 15 minutes. The carbon dioxide collected in the trap was precipitated as barium carbonate. Under these conditions quantitative decarboxylation was obtained and the blank was very small.

The carboxylation of *o*-coumaric acid was tested on a chemically synthesized, specifically labelled sample and proved valid. No coumarin-4-C^{14} was available for a direct check of the validity of the alkali fusion step, but fusion of coumarin-2- and -3-C^{14} under the same conditions yielded in each case inactive salicylic acid, precluding any rearrangements involving the side chain.

Measurement of Radioactivity

All organic compounds were subjected to wet oxidation (28) and barium carbonate samples to acidification, for the recovery of carbon dioxide. This was counted in the gas phase with a Nuclear-Chicago Model 6000 dynamic condenser electrometer incorporating an ion chamber.

Results and Discussion

Comparison of Labelled Compounds as Coumarin Precursors

In the early stages of this investigation a number of experiments were done using *Melilotus officinalis*. Under our conditions it was found impossible to grow this species satisfactorily in the wintertime, and various feeding experiments carried on over a 2-year period gave inconsistent results with regard to coumarin formation. However, two of the findings are probably of significance and will be reported briefly. First, when whole plants were exposed to $C^{14}O_2$ and subsequently allowed to metabolize in the light at 23° the specific activity of the coumarin continued to increase up to the termination of the experiment at 90 hours. The specific activities were 0.00, 0.48, and 1.7 μc/mmole at 1, 24, and 90 hours, respectively. These results differ from those of Kosuge and Conn (10) with *M. alba*, which showed a peak specific activity after a metabolic period of 24 hours. Second, cinnamic acid proved to be a consistently good precursor of coumarin in comparison with a number of other compounds tested, C^{14}-dilutions of the order of 200 to 400 being obtained in the best experiments.

Preliminary studies carried out on *H. odorata* indicated that cinnamic acid was also a relatively good coumarin precursor in this species. Two experiments were then carried out in which a number of possible precursors were compared; the results are presented in Table I. In the first of these (A), greenhouse-grown plants were employed, and the coumarin recovered by extraction with 80% ethanol. In the second (B), plants cultivated in artificial growth chambers were used and the coumarin was isolated by steam distillation. The metabolic periods after absorption of the radioactive solutions were 24 and 7.5 hours, respectively. In all cases significantly lower dilutions were obtained in experiment A. This result can be attributed in part to differences in the amounts of endogenous coumarin, which would have diluted the radioactive coumarin formed in experiment B an average of 6 times more than that formed in experiment A. In addition, as will be seen later, the difference in the duration of the experiments was undoubtedly a contributing factor. In both experiments the best precursors of coumarin were *o*-coumaric, cinnamic, and shikimic acids and L-phenylalanine. However, in experiment A, acetate and especially melilotic acid appear to have been relatively much more efficiently utilized than in experiment B. In view of the fact that coumarin from the latter experiment was subjected to the more rigorous purification it is believed that the results of this experiment are less subject to possible contamination errors and are, therefore, more reliable. To check this point another experiment was run, under similar conditions to those of experiment B, in which cinnamic, *o*-coumaric, and melilotic acids were compared. The dilutions were 340, 930, and 5700 respectively. Clearly melilotic acid is a much less efficient coumarin precursor than cinnamic acid.

The figures in Table I suggest that the shikimic acid – phenylpropanoid acid pathway predominates in coumarin biosynthesis in *H. odorata*. The relatively efficient utilization of cinnamic and *o*-coumaric acids implies that the former undergoes hydroxylation ortho to the side chain during the process. It is of interest that acetate–carbon is incorporated measurably into coumarin, better

TABLE I

Conversion of labelled compounds to coumarin in *H. odorata*

	Compound administered		Coumarin isolated					
	Dose rate,* μmoles/g fresh wt.	Specific activity,* μc/mmole	Amount, mg/g fresh wt.		Specific activity, mμc/mmole		Dilution	
Name	(A)	(A)	(A)	(B)	(A)	(B)	(A)	(B)
o-Coumaric acid-1-C^{14}	8.0	50	0.23	0.56	5,590	43	9	1,200
Cinnamic acid-3-C^{14}	7.7	50	0.25	1.8	247	43	202	1,200
Shikimic acid-R-C^{14}	12.0	40	0.25	1.7	210	17	190	2,900
L-Phenylalanine-R-C^{14}	8.9	51.5	0.19	1.05	108	11	480	4,500
Acetic acid-1-C^{14}	10.8	41	0.31	0.98	50	3.9	820	13,000
Salicylic acid-$C^{14}OOH$	—	—	—	1.75	—	2.7	—	19,000
Acetic acid-2-C^{14}	—	—	—	1.9	—	2.2	—	23,000
o-Tyrosine-3-C^{14}	—	—	—	1.85	—	2.0	—	25,000
Ferulic acid-2-C^{14}	—	—	—	1.7	—	1.3	—	38,000
Melilotic acid-1-C^{14}	10.5	41.5	0.20	1.55	62	0.6	670	80,000

*In experiment (B) the dose rate of the precursor was maintained constant at 4 μmoles/g fresh weight of plant, and the specific activity of the precursor at 50 μc/mmole.

than three of the phenylpropanoid compounds tested. This does not necessarily imply the existence of Birch–Donovan aromatization, as the activity may reside in the side chain, but that possibility should be borne in mind as a possible minor pathway. The utilization of salicylic acid to about the same degree also leaves open the question of a $C_6,C_1 + C_2$ condensation such as has been suggested to occur in coumarin formation (5) as well as in lignification (14, 29).

In experiment B an attempt was made after each feeding to recover enough free *o*-coumaric acid for estimation of its C^{14}-content. This proved difficult at first because the compound was present only in microgram amounts, but in three cases enough was recovered to obtain what was considered a reliable value. In most cases, during continuous ether extraction of the organic acids from aqueous solution, crystals of *o*-coumaryl glucoside separated in the receiver, and this material too was analyzed for C^{14}. The results are shown in Table II. Except in the salicylic acid and *o*-tyrosine experiments, the specific

TABLE II

Conversion of labelled compounds to *o*-coumaric acid and *o*-coumaryl glucoside in *H. odorata*

	Specific activity, mμc/mmole		Dilution	
Compound administered	*o*-Coumaric acid	*o*-Coumaryl glucoside	*o*-Coumaric acid	*o*-Coumaryl glucoside
o-Coumaric acid-1-C^{14}	—	8,470	—	5.9
Cinnamic acid-3-C^{14}	15,600	—	3.2	—
Shikimic acid-R-C^{14}	—	1,140	—	44
L-Phenylalanine-R-C^{14}	—	555	—	90
Acetic acid-1-C^{14}	—	505	—	99
Salicylic acid-$C^{14}OOH$	—	3.4	—	15,000
o-Tyrosine-3-C^{14}	80	7	600	7,000
Ferulic acid-2-C^{14}	—	36	—	1,400
Melilotic acid-1-C^{14}	480	—	100	—

activity of the *o*-coumaryl glucoside is much higher than that of the corresponding coumarin sample. As the amounts of the two compounds isolated were of comparable magnitude, the total C^{14} entering the glucoside from cinnamic

acid during the period studied was also much greater than that being incorporated into coumarin. These findings parallel those of Kosuge and Conn (10), who fed acetate, phenylalanine, and cinnamic acid to *M. alba*. The results of our experiment appear to indicate that acetate was almost as well utilized as L-phenylalanine in the synthesis of *o*-coumaryl glucoside. The C^{14} was in the aglycone, not in the sugar, because it was the *o*-coumaric acid from hydrolysis of the glucoside with emulsin which was actually combusted for counting. However, it has not been possible to reproduce this result, and its significance remains questionable.

The small size of the free *o*-coumaric acid pool in the experimental plants (< 1 mg) has resulted in a very low dilution of the C^{14} in the administered cinnamic acid. The C^{14} of melilotic acid and *o*-tyrosine was diluted 30 and 150 times more although the melilotic acid pool was even smaller, and that of *o*-tyrosine probably non-existent. These results imply that melilotic acid is not an intermediate in the biosynthesis of coumarin or *o*-coumaric acid, an inference which receives further support from the time studies to be reported below. A more probable alternative is that melilotic acid is formed from coumarin (10), or is a reduction product of *o*-coumaric acid.

Degradation of Coumarin

The two independent degradation schemes (Fig. 1) outlined in the experimental section have been applied to coumarin samples recovered after feeding

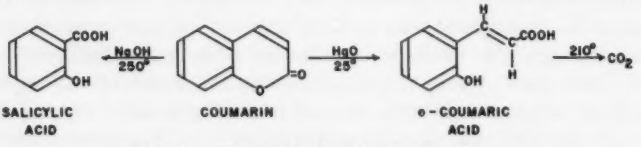


FIG. 1. Coumarin degradation reactions.

cinnamic acid-1- and -3-C^{14} and *o*-coumaric acid-1-C^{14}. The data from these degradations are listed in Table III. The figures obtained after feeding the

TABLE III

Degradation of coumarin from *H. odorata* fed C^{14}-labelled cinnamic and *o*-coumaric acids

Precursor	Specific activity, mμc/mmole			% of C^{14} recovered
	Coumarin	Salicylic acid from coumarin	C-1 of *o*-coumaric acid from coumarin	
o-Coumaric acid-1-C^{14}	11.5	—	9.39	82
Cinnamic acid-1-C^{14}	148	—	139	94
Cinnamic acid-3-C^{14}	73	40.4	—	55
	730	740	—	101
	873	769	—	88

carboxyl-labelled *o*-coumaric and cinnamic acids suggest only a low degree of randomization, which may arise as a result of limited decarboxylation.

The recovery of C^{14} as salicylic acid in the first cinnamic acid-3-C^{14} experiment is low, but in the two subsequent degradations the figures are consistent with

those obtained with the carboxyl-labelled precursors. This one low result would thus appear to be an unexplained anomaly. Omitting it, the average recovery of C^{14} from the predicted position of coumarin was 91%. The incorporation of the carbon skeleton of these phenylpropanoid acids into coumarin as a unit is clearly the only important biosynthetic pathway here.

Time Studies

The effect of varying metabolic periods on the radioactivity of several phenolic constituents of *Hierochloë*, including coumarin and structurally related compounds, has been studied after administration of cinnamic acid-3-C^{14}. Figure 2 shows the changes in specific activity of coumarin isolated in two

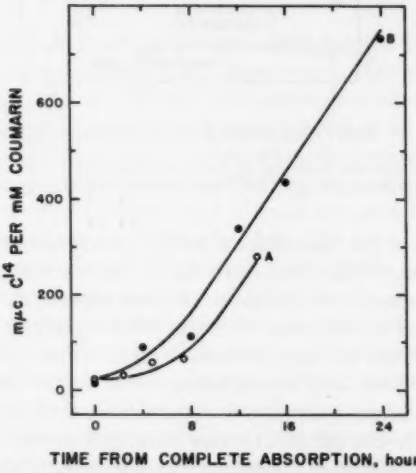


FIG. 2. Change of specific activity of coumarin with time after feeding cinnamic acid-3-C^{14} to shoots of sweet grass (*Hierochloë odorata*).

experiments of this type, one of which was run up to 24 hours. In each case there is a definite lag phase persisting until about 8 hours from the complete absorption of the precursor. The curve for experiment B shows that from that time until at least 24 hours the increase in specific activity is rectilinear. As there is no large change in total coumarin over this period, a curve showing total C^{14}-content of coumarin is almost identical in form.

The specific activities of four free phenylpropanoid acids and *o*-coumaryl glucoside from this same experiment are plotted in Fig. 3. Of these, ferulic and *p*-coumaric acids have a hydroxylation pattern differing from that of coumarin and are included incidentally. The picture for these two acids generally resembles that found in *Salvia splendens* by McCalla and Neish (25), when allowance is made for the similar amounts of the two acids present in *Hierochloë*. All four free acids exhibit a peak in specific activity at or before 16 hours, and all but melilotic have attained a relatively high specific activity in 0 to 4 hours. The slow and slight accumulation of C^{14} in melilotic acid again suggests that it is a secondary metabolic product of cinnamic acid.

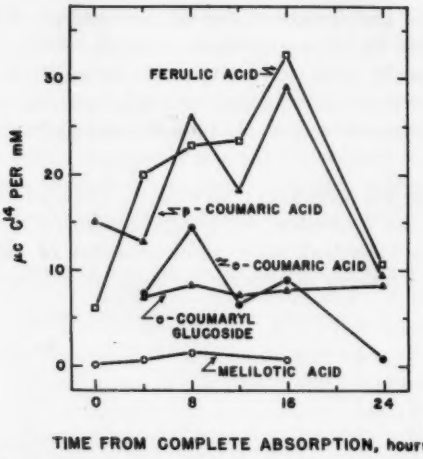


FIG. 3. Change of specific activity of free phenolic acids and *o*-coumaryl glucoside with time after feeding cinnamic acid-3-C^{14} to shoots of sweet grass (*Hierochloë odorata*).

A clearer picture of the distribution of the active carbon can be obtained from an examination of the total activity in the various constituents. The amount of free *o*-coumaric acid recovered in these experiments averaged 0.5 mg; this is a minimum value but it can be considered correct as to order of magnitude. At 8 hours, therefore, free *o*-coumaric acid contained ca. 0.05 μc of C^{14}. At the same time melilotic acid accounted for only about 0.0015 μc. Coumarin, in spite of the much larger quantity, contained only 0.04 μc, about the same as free *o*-coumaric acid, but after 24 hours this had increased to 0.23 μc. In contrast, *o*-coumaryl glucoside contained virtually identical quantities (1.5 μc) of C^{14} at 8 and 24 hours, amounting to 16% of the total administered radioactivity. It is clear from these data that the largest reservoir of *o*-hydroxylated phenylpropanoid material throughout the period of the experiment was *o*-coumaryl glucoside. This is especially true in the period closely following uptake of the cinnamic acid. No glucoside was recovered from the "zero"-hour plants in this experiment, but in a repeat it was found that the C^{14}-content of the glucoside was even higher at 0 than at 4 hours. These results show unequivocally that cinnamic acid administered to *H. odorata* undergoes rapid ortho-hydroxylation and that glucoside formation also occurs rapidly, with a resulting large accumulation of C^{14} in *o*-coumaryl glucoside even while absorption of the precursor is still in progress.

The long time lag shown in Fig. 2 is not easy to explain, as knowledge of the reactions involved is presently incomplete. It could be a manifestation of two consecutive slow reactions with comparable reaction rates. The rapid formation of *o*-coumaric acid is proof of the facility with which the *o*-hydroxyl is introduced into cinnamic acid; consequently any such rate-determining reactions must lie beyond this point, and may be associated with the *trans–cis* inversion which must precede lactonization.

The Role of Glucosides in Coumarin Formation

The above findings indicating a high rate of conversion of cinnamic acid to *o*-coumaryl glucoside have raised the question whether the latter compound is an intermediate in the biosynthesis of coumarin. To test this possibility a sample of radioactive *o*-coumaryl glucoside isolated after administration of cinnamic acid-3-C^{14} to *Hierochloë* and a sample of cinnamic acid-3-C^{14} (0.062 mmole of each) were fed to 15-g lots of shoots, and the plants allowed to metabolize in the greenhouse for about 48 hours. The plants were then worked up as before and coumarin isolated. The C^{14} dilution during conversion of cinnamic acid to coumarin was 315, and that for *o*-coumaryl glucoside was 435. The two compounds were, therefore, comparable in efficiency as precursors of coumarin.

In spite of this result it would be premature to suggest that cinnamic acid is necessarily converted to coumarin via *o*-coumaryl glucoside. Isolation procedures for glucosides are notoriously productive of artifacts (30), and in the work to date few special precautions have been taken to guard against that eventuality. Repeated difficulties encountered in the isolation of an appreciable part of the phenolic glycoside material, both by ether extraction and the use of an anion exchange resin, imply that a more complex condition may exist in this species with regard to glucosides than has been previously thought. Not all of the glucoside of *o*-coumaric acid may exist in the plant as the free acid, rather than in some combination as, for example, an ester or depside.

Further evidence concerning the relation of the glucoside to coumarin should be forthcoming from an extension of the time studies reported here to periods of several days. This work is now in progress, but because of difficulties in maintaining plants in healthy condition for the duration of such an experiment no conclusive results have yet been obtained. In the meantime the matter of biosynthetic relationships between the phenolic acid glucosides and coumarin must remain in abeyance.

Acknowledgments

Synthetic *o*-coumaryl glucoside and coumarin-3-C^{14} were generously donated by Drs. E. E. Conn and T. Kosuge of the University of California, Davis, who were also kind enough to allow the authors to read their manuscript before its publication. The authors are also grateful to Dr. R. P. Knowles of the Canada Agriculture Research Laboratory, Saskatoon, for locating a source of wild *Hierochloë odorata*, and to Dr. J. E. R. Greenshields, formerly of the C.A.R.L., for gifts of *Melilotus* seed. Thanks are due to Dr. Greenshields and to Dr. A. C. Neish for several valuable discussions. Messrs. J. P. Shyluk and John Dyck have contributed their usual high standard of technical assistance.

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Addendum

Since the submission of this paper a recent publication by Weygand and Wendt (Z. Naturforsch. **14b**, 421 (1959)) has come to our attention. They have reported that coumarin is synthesized by cultures of *M. officinalis* root tissue from phenylalanine and glucose, but not from acetate. Unfortunately no data are presented from which the efficiency of utilization of these precursors can be calculated. Extensive degradation studies on coumarin formed from glucose-1-C^{14} have led these authors to suggest that coumarin arises via the shikimic acid pathway, a conclusion identical with that presented here on the basis of more direct evidence.

THE BIOSYNTHESIS OF ERYTHRITOL AND GLYCEROL BY TORULOPSIS MAGNOLIAE. STUDIES WITH C^{14}-LABELLED GLUCOSE[1]

J. F. T. SPENCER AND P. A. J. GORIN

Abstract

D-Glucose-1-C^{14}, D-glucose-2-C^{14}, D-glucose-3,4-C^{14}, and D-glucose-6-C^{14} were dissimilated aerobically by washed cells of *Torulopsis magnoliae*. The major products formed were erythritol and glycerol.

A method for the stepwise degradation of erythritol, after oxidation with *Acetobacter suboxydans* to L-erythrulose, is described. The distribution of radioactive carbon in the erythritol suggests that it is formed by a complex series of reactions in which enzymes of the transaldolase and transketolase type play a major part to give a four-carbon fragment which is then reduced to the tetritol. Enzymes of the Embden–Meyerhof pathway are probably concerned to a lesser extent.

Introduction

Torulopsis magnoliae, strain H_3C, produces high yields of glycerol and erythritol (1, 2). Glycerol is known to be formed in *Saccharomyces cerevisiae* and other organisms via the Embden–Meyerhof pathway, but the reactions leading to the formation of erythritol by yeast have not been studied.

The reactions postulated to occur in the intermediary metabolism of pentoses (3), in photosynthesis (4), when four-carbon chains are formed as by-products, and in the breakdown of hexulose (5), may be responsible for the synthesis of the carbon skeleton of erythritol. These mechanisms do not, however, account completely for erythritol biosynthesis. The results of studies of the dissimilation of C^{14}-labelled glucose and of the labelling of the polyols formed are reported, and possible biosynthetic pathways for erythritol formation are suggested.

Experimental

Preparation of Labelled Compounds

Glucose-1-C^{14}, glucose-2-C^{14}, and glucose-6-C^{14} were supplied by Dr. A. C. Neish of this laboratory. Glucose-3,4-C^{14} was prepared from C^{14}-labelled bicarbonate by the method of Wood *et al.* (6).

D-Erythritol-4-C^{14}* was prepared by sodium borohydride reduction of D-erythrose-4-C^{14} obtained by lead tetraacetate oxidation of D-glucose-6-C^{14} (7).

D-Erythritol-1-C^{14} was prepared by the addition of potassium cyanide-C^{14} to D-glyceraldehyde by a method suggested by Isbell (8). The D-erythrono-γ-lactone formed was reduced by sodium borohydride in methanol (9) to

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*Although erythritol normally has the meso-configuration it is referred to as D-erythritol throughout the paper, since C^{14} in the molecule confers asymmetry to it and also because the discussion is simplified by regarding the polyol in this way.

D-erythritol. The latter was degraded stepwise by the method described in this paper and found to have 91% of total activity in C-1 and 9% in C-4 of the molecule.

Cultural Methods

Torulopsis magnoliae, strain H_3C, was isolated from honey and maintained on slants of a medium containing 60% honey, 0.25% yeast extract, and 2% agar. Cultures for washed cell preparations were grown in a medium containing 20% glucose, 1.3% yeast extract, and 0.3% urea, incubated 48 hours at 30° C on a rotary shaker, eccentricity 1 in., operated at 230 r.p.m. The cells were recovered by centrifugation and washed with 0.8% potassium chloride solution.

Acetobacter suboxydans, strain G12, was grown in a medium containing 5.0% glycerol and 0.5% yeast extract, incubated 16 hours at 30° C on a rotary shaker, eccentricity 1 in., at 230 r.p.m. The cells were recovered by centrifugation, washed twice with 0.8% potassium chloride solution, and resuspended in 5 to 10 ml water.

Dissimilation of Labelled Substrates

The dissimilation of the labelled glucose samples was done in 500-ml Erlenmeyer flasks stoppered and fitted with inlets and outlets through which carbon-dioxide-free air was passed. The carbon dioxide produced during fermentation was absorbed in carbonate-free alkali solution. The fermentation flasks were incubated at 24° C on a rotary shaker of ½-in. eccentricity operated at 230 r.p.m. At higher temperatures less erythritol was formed. When glucose dissimilation was complete, as determined by estimation of reducing sugar in a control flask containing unlabelled glucose, the cells were removed by centrifugation, and the glycerol and erythritol were separated by partition chromatography on cellulose columns (10).

Oxidation of Erythritol to L-*Erythrulose with A. suboxydans*

The cells from 100 ml of broth were placed in a 100-ml round-bottomed flask containing up to 600 mg erythritol in 5 to 10 ml water. The flask was shaken on a Burrell wrist action shaker at room temperature. Oxidation of erythritol to L-erythrulose was complete in 3 to 4 hours. The reaction was followed by chromatography of small aliquots on strips of Whatman No. 1 filter paper, which were developed in a mixture of ethyl acetate, acetic acid, and water (9:2:2 v/v) in 200×25 mm culture tubes. The spots were detected with alkaline silver nitrate (11). On completion of the reaction the cells were removed by centrifugation and the L-erythrulose selectively degraded as described below.

Analytical Methods

Reducing sugars were determined colorimetrically with the alkaline copper reagent of Somogyi (12) and the arsenomolybdate reagent of Nelson (13).

Polyhydric alcohols were estimated by oxidation with periodic acid and colorimetric determination of the formaldehyde formed with chromotropic acid reagent according to the method described by Neish (14).

Glycerol and *erythritol* were separated on a preparative scale by partition chromatography on cellulose columns using *n*-butanol one-quarter saturated with water as developing solvent.

Fermentation acids were separated by partition chromatography on silicic acid – water columns (14).

C^{14} determinations were carried out by oxidizing the compound to CO_2 with a Van Slyke mixture (16) and then measuring the radioactivity with a Nuclear-Chicago Model 6000 dynamic condenser electrometer, incorporating an ion chamber.

Degradation of Fermentation Products

Glycerol

Glycerol was oxidized with periodic acid to formaldehyde and formic acid. The formaldehyde was precipitated as its dimethone derivative (15) and the formic acid was oxidized to carbon dioxide with mercuric oxide and phosphoric acid (16).

Erythritol

The procedure described above for glycerol was used to determine the radioactivity in C-1+C-4.

To obtain data which could be used to complete the determination of the activity in each carbon atom of erythritol, radioactive erythrulose, obtained by oxidation of a portion of the erythritol with *A. suboxydans*, was degraded as follows: About 60.0 mg of erythrulose was treated with aqueous phenylhydrazine acetate at 40° C to yield L-erythrulose phenylosazone (17) which could, in turn, be degraded to mesoxaldehyde phenylosazone and formaldehyde by oxidation with sodium periodate in aqueous ethanol (18). The mesoxaldehyde phenylosazone was completely precipitated from the reaction mixture by addition of excess water and was recrystallized from ethanol. The formaldehyde in the aqueous portion was precipitated as its dimethone derivative. The derivative was obtained in good yield by distillation *in vacuo* of the ethanol. Filtration and recrystallization from ethanol afforded a pure product. The specific activities of (C-2+3+4) and C-1 of the D-erythritol molecule were thus obtained.

It has been shown that a 4 molar excess of lead tetraacetate reacts with L-erythrulose in aqueous acetic acid to the extent of 1.88 moles (steady state) in 1.5 hours (19). Since this result suggested that glycolic acid (C-3+4) was formed, a method was developed for its isolation, which led to a complete determination of the activities in the individual carbon atoms in the erythritol chain. In a typical degradation erythrulose (162 mg) in water (2 ml) and acetic acid was treated for 3 hours with lead tetraacetate (2.0 molar equivalents) in 25 ml acetic acid. Oxalic acid dihydrate (0.15 g) in acetic acid (5 ml) was then added and the precipitate collected. The filtrate was evaporated to dryness *in vacuo*, the residue dissolved in water, and Amberlite IR-120 added. The solution was evaporated and glycolic acid was obtained by three extractions with boiling ether. The extract was evaporated to 0.5 ml and slow

cooling to −30° C yielded 10 mg crystalline glycolic acid, which was sufficient for the determination of specific activity.

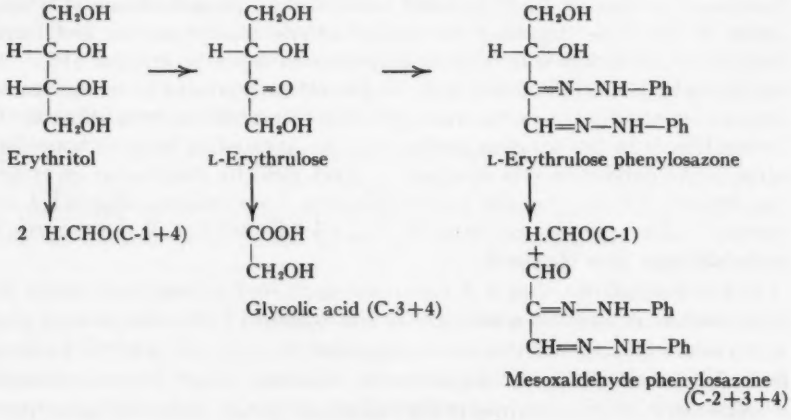
Degradation of authentic D-erythritol-4-C^{14} by these methods indicated (Table I) that the error in the method was about 10% and that there was no

TABLE I

Degradation of D-erythritol-4-C^{14}

	No. of carbon atoms	Product, mμc/mmole	C^{14}, % of total in erythritol
Erythritol	1+2+3+4	75.7	—
Formaldehyde-dimethone (from erythritol)	1+4	9.11	102
Erythrulose phenylosazone	1+2+3+4	16.9	90
Mesoxaldehyde phenylosazone	2+3+4	18.0	89
Formaldehyde dimethone (from erythrulose phenylosazone)	1	0	0
Glycolic acid	3+4	141	94
Formaldehyde-dimethone (from reduced erythrulose)	1+4	8.7	97

rearrangement of the carbon skeleton of erythritol during oxidation by *A. suboxydans*. The degradation scheme is summarized in the following series of reactions:



Results and Discussion

Yields and specific activities of the products of glucose dissimilation are shown in Table II. Four to ten per cent of the total radioactivity was incorporated into the erythritol. Small quantities were incorporated into succinic acid, but the yield of this compound was so small that it was not investigated further. Fifty-five to seventy per cent of the activity appeared in the carbon dioxide and cell carbon.

TABLE II

Products of glucose dissimilation by washed cells of *T. magnoliae*

	Product, mmoles	Specific activity (mμc/mmole) of products from: Glucose-1-C^{14}	Glucose-2-C^{14}	Glucose-3,4-C^{14}	Glucose-6-C^{14}
Initial glucose	16.7	600	600	5.4	568
Final glucose	0.45	—	—	—	—
Glycerol	0.62	158	66.4	—	272
Erythritol	2.77	144	123	7.7§	390
Succinic acid	0.04	234	228	—	345
Cell carbon†	97.2	32.0	37.9	—	39.2
Carbon dioxide	39.2	100.8	66.1	—	52.2
% contribution‡ of the labelled carbon atom to erythritol		30	50	95	68

*From dissimilation of glucose-1-C^{14}.
†Includes cells added at the beginning of the fermentation.
‡$\frac{\text{Specific activity of erythritol} \times 4}{\text{Specific activity of glucose} \times 6} \times 100$.
By difference, carbon-5 of glucose was incorporated to the extent of 62%.
§Very little glucose-3,4-C^{14} was available. The yield of erythritol was sufficient for determination of specific activity, but not for stepwise degradation and determination of the activity in each carbon atom.

The labelling in glycerol (Table III) is as predicted by known biosynthetic pathways (20, 21). Radioactivity from carbons 1 and 6 of glucose appeared in

TABLE III

Distribution of carbon-14 as % of total activity in the product

	Glucose-1-C^{14}	Glucose-2-C^{14}	Glucose-6-C^{14}
Glycerol—CH_2OH	97	55	99
—CHOH	3	45	1
Erythritol			
C-1	24	44	1
C-2	17	10	20
C-3		30	0
C-4	59	10	80

NOTE: Carbon-1 determined directly. Carbons 2, 3, and 4 calculated by difference.

the terminal carbons of glycerol, and activity from carbon-2 of glucose appeared in the central carbon, as predicted by the Embden–Meyerhof scheme. Activity from carbon-2 of glucose which appeared in the terminal carbons of glycerol may be explained by the rearrangement of the original carbon chain of glucose prior to resynthesis of hexose by transaldolase (Fig. 1).

Several factors must be considered in deducing pathways leading to the formation of erythritol. These are the formation by the organism of three-carbon chains (glycerol), the relatively high specific activity of carbon dioxide formed from glucose-1-C^{14} compared with that obtained from glucose labelled in other positions, and the high incorporation of C^{14} into erythritol formed from glucose-6-C^{14} and glucose-3,4-C^{14}.

From these observations a number of reactions may be deduced which provide a possible explanation of the distribution of radioactivity in the

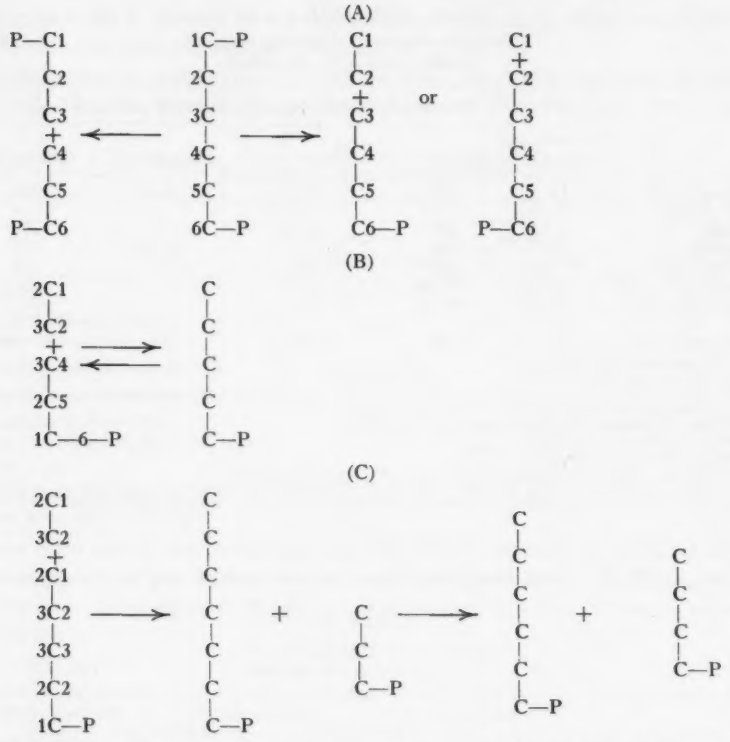


FIG. 1. Hypothetical carbon skeleton changes associated with erythritol formation by *T. magnoliae*.*

erythritol molecule (Fig. 1). The formation of glycerol from glucose and the formation of carbon dioxide of relatively high specific activity from glucose-1-C^{14} show that two of the reactions of Fig. 1(A), leading to the formation of three- and five-carbon chains, are probable. The relatively high specific activity of erythritol formed from glucose-6-C^{14} suggests that the third reaction of Fig. 1(A), catalyzed by transketolase and leading directly to the formation of a four-carbon chain, is important. The three- and five-carbon chains mentioned previously can act as acceptors in the latter reaction, and can thus take part in the longer series of transketolase-and transaldolase-catalyzed reactions (Figs. 1(B) and 1(C)) which lead to the incorporation of activity from carbons 1 and 2 of glucose into erythritol by way of a seven-carbon intermediate. The lower percentage incorporation of activity into erythritol from carbons 1 and 2 of glucose (Table II) suggests that the greater part of the erythritol was formed directly from carbons 3–6 of glucose.

The hypothetical distribution of radioactivity in erythritol from carbons 1, 2, and 6 of glucose (Fig. 2) according to the above reactions agrees reasonably

*Numbers refer to the original carbon atom in the glucose molecule. They are omitted in the later stages of some reactions, because of the large number of combinations possible. P is a phosphate group.

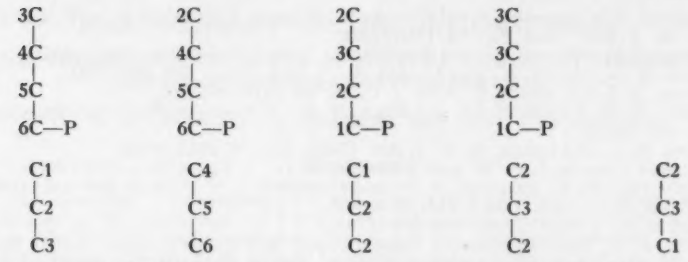


FIG. 2. Hypothetical labelling of three- and four-carbon chains formed during erythritol formation by *T. magnoliae* according to the reactions in FIG. 1.*

well with the experimental results. Carbon-1 and carbon-6 of glucose appear in carbon-4 of erythritol as predicted, and likewise carbon-2 of glucose appears in carbons 1 and 3 of erythritol. Some radioactivity appears in other positions, for which the foregoing schemes do not account. This could not be accounted for by recycling of erythritol during the fermentation, which was shown not to occur (Table IV). Since the osmosphilic yeasts produce traces of low molec-

TABLE IV

Distribution of radioactivity in labelled erythritol before and after incubation in the presence of *T. magnoliae* fermenting glucose (% of total in product)

	D-Erythritol-1-C^{14}		D-Erythritol-4-C^{14}	
Carbon No.	Before	After	Before	After
1	91	84	0	3
2	0	0	0	0
3	0	0	0	0
4	9	16	100	97

ular weight compounds (1), other reactions not included in these schemes may contribute to some extent to the formation of erythritol. However, the most important reactions leading to erythritol formation are probably those catalyzed by the enzymes of the Embden–Meyerhof pathway, by transaldolase, and by transketolase.

Acknowledgments

The authors wish to thank Dr. F. Lotz, Dr. W. C. C. McMurray, and Dr. D. V. Cormack, of the Saskatchewan Cancer and Medical Research Unit, for valuable assistance in the synthesis of glucose-3,4-C^{14}. The technical assistance of Miss S. Lubin, Mr. N. R. Gardner, and Mr. J. Dyck is gratefully acknowledged.

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*Numbers refer to the original carbon atoms in the glucose molecule.

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BIOCHEMISTRY OF THE USTILAGINALES

XIII. OBSERVATIONS ON THE STRUCTURE AND BIOSYNTHESIS OF 4-*O*-β-D-MANNOPYRANOSYL-D-ERYTHRITOL[1]

P. A. J. GORIN, R. H. HASKINS, AND J. F. T. SPENCER

Abstract

D-Glucose-6-C^{14}, D-erythrose-4-C^{14}, and D-erythritol-4-C^{14} were used as precursors in the biosynthesis of 4-*O*-β-D-mannopyranosyl erythritol by *Ustilago* sp. PRL 627 and the patterns of C^{14} labelling in the products determined. The disaccharide appears to be formed by direct transformation of D-glucose to a D-mannose unit which is then attached to C-4 of D-erythritol.* The labelling of the D-erythritol† portion shows that 80% of C-4 of the molecule is derived from the 6-position of D-glucose.

Introduction

In previous publications (1, 2) the production of various metabolites formed in aerobic submerged cultures of *Ustilago* sp. PRL 627 has been described. The two main products formed consist of a water-soluble disaccharide containing D-mannose and erythritol and an extracellular 'oil'. The latter was identified tentatively as a mixture of mannopyranosyl erythritol residues to which acetic and several saturated and unsaturated acids are joined by ester linkages. The free disaccharide has been shown to consume in water 4 molar equivalents of sodium periodate liberating 2 moles of formic acid and 1 mole of formaldehyde (2), thus indicating that it consists of a D-mannopyranosyl residue glycosidically attached to a terminal carbon atom of erythritol. The specific rotation (−106°) of the trialdehyde at completion of the oxidation indicated that the glycosidic linkage configuration was β (3). Thus the disaccharide could be assigned either structure I or II, which differ in the linkage position in the erythritol moiety. By means of C^{14} tracer and biochemical oxidation techniques the compound is shown to possess the 4-*O*-β-D-mannopyranosyl-D-erythritol structure (II) and the probable mode of biosynthesis of this compound is determined. The biosynthesis of erythritol and D-mannitol, which are minor products in some of the fermentations with radioactive sugars, are also considered.

Experimental

Preparation of C^{14}-Labelled Compounds

D-Glucose-6-C^{14} was supplied by Dr. A. C. Neish of this laboratory and D-erythrose-4-C^{14} and D-erythritol-4-C^{14} were prepared using the procedure of Perlin and Brice (4).

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*This finding shows that the erythritol unit of the disaccharide possesses the 4-*O*-D- (or 1-*O*-L-) configuration. The approved nomenclature for this compound is 1-*O*-β-D-mannopyranosyl-L-erythritol, but in this publication it is given the alternative name for the sake of convenience in the discussion.

†Although erythritol normally has the meso-form it is referred to throughout this paper as D-erythritol since the C^{14} in the molecule confers asymmetry.

Production of Mannopyranosyl Erythritol-C^{14}

The fungus *Ustilago* sp. PRL 627 was grown on a medium containing glucose as described by Haskins, Thorn, and Boothroyd (1). The precursor containing C^{14} (ca. 75 mg; ca. 10 μc) was added to an aqueous solution of glucose (20 g in 100 cc) which was assayed for C^{14}, nutrient salts were added, and the solution was sterilized by filtration. The inoculum was added and, after the fermentation was complete, barium hydroxide (25 g) was added and dissolved by heating the solution on a steam bath for 1 hour. After the ester linkages were thus destroyed, barium was removed by bubbling carbon dioxide through the solution, which was filtered and evaporated to a syrup. This was resolved into its components by chromatography on a cellulose column (5) using *n*-butanol saturated with water as eluant. The main product from the column was mannopyranosyl erythritol, but with some of the fermentations small amounts of erythritol and D-mannitol were also found.

Addition of Various Alcohols to Fermentation

In four separate experiments D-mannitol (5.0 g), ribitol (4.0 g), glycerol (2.4 g), and ethanol (1.2 g) were added to the medium containing 20 g glucose prior to inoculation. Paper chromatographic examination of the solution after fermentation using *n*-butanol–ethanol–water (40:11:19 v/v) as solvent and ammoniacal silver nitrate (6) as spray reagent showed that in each case mannopyranosyl erythritol production had been inhibited almost completely. No compounds with R_f values corresponding to alcohol mannoside derivatives were present. However, erythritol was present in each fermentation and was the only major product apart from the unfermented alcohol.

Stepwise Degradation of Mannopyranosyl Erythritol-C^{14}

(*i*) *Mannopyranosyl erythritol* was oxidized with excess aqueous sodium periodate to yield the formaldehyde derived from C-1 of the D-erythritol residue. The formaldehyde was isolated as its dimethone derivative (7).

The disaccharide was hydrolyzed with *N* sulphuric acid at 100° C for 18 hours, the solution neutralized ($BaCO_3$), filtered, and evaporated to a mixture of products. This was separated to give mannose and erythritol by chromatography on a cellulose column using *n*-butanol half-saturated with water as the mobile phase. Each component was crystallized once from ethanol.

(*ii*) D-*Mannose* was degraded with sodium periodate and the formaldehyde produced (C-6) converted to its dimethone. The C^{14} in C-1 and C-6 was measured in a similar manner by periodate oxidation of the D-mannitol derived by sodium borohydride reduction (8), C-1 being obtained by difference.

(*iii*) *Erythritol* was degraded by the method described previously (9) giving C-1 separately and C-4 by difference.

(*iv*) D-*Mannitol* was isolated by crystallization from methanol; m.p. and mixed m.p. 166–168° C. C-1 plus C-6 were determined as described for D-mannose.

Carbon-14 determinations were made by oxidation of the compounds with Van Slyke reagent to carbon dioxide and measurement of the radioactivity

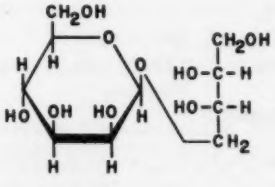
with a Nuclear-Chicago Model 6000 dynamic condenser electrometer, incorporating an ion chamber.

Attempted Oxidation of 4-O-β-D-Mannopyranosyl-D-Erythritol with Acetobacter suboxydans

To a solution of mannopyranosyl erythritol (50 mg) in water (10 cc) washed cells of *Acetobacter suboxydans* were added (9). At intervals up to 20 hours, aliquots of the solution were taken and examined on a paper chromatogram using *n*-butanol–ethanol–water (40:11:19 v/v) as solvent and ammoniacal silver nitrate and *p*-anisidine hydrochloride (10) as spray reagents. No positive reaction with the latter spray was noted, indicating that a ketose derivative had not been formed.

Results and Discussion

When a straight chain polyol with the D-erythro-configuration is oxidized by *Acetobacter suboxydans* or *Acetobacter xylinum*, the hydroxy group adjacent to the terminal hydroxy group is converted to a ketone according to the Bertrand–Hudson rule (11, 12). Structure I would then be oxidizable and structure II, which has the L-erythro-configuration relative to the free terminal hydroxy group would be unaffected. It was shown that the metabolite was unchanged after attempted *Acetobacter suboxydans* oxidation, thus indicating structure II.



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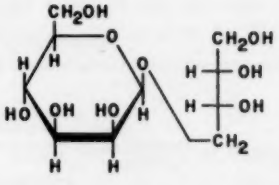

II

A more definite proof of the structure was obtained, however, by using the C^{14} tracer technique. D-Glucose-6-C^{14}, D-erythrose-4-C^{14}, and D-erythritol-4-C^{14} all furnished mannopyranosyl erythritol which had little or no specific activity in the free terminal carbon atom of the erythritol (Table II). When erythritol was obtained from each sample by acid hydrolysis it was oxidized with *Acetobacter suboxydans* (13) to give L-erythrulose. (This oxidation has been recently tested by converting a known sample of D-erythritol-4-C^{14} to L-erythrulose-1-C^{14}, with no dilution or randomization of C^{14} activity (9).) Stepwise degradation of the derived L-erythrulose samples (9) obtained from the disaccharide showed that the D-erythritol molecules were unlabelled in C-1 and labelled in the C-4 position. Comparison of the specific activities of the carbons in the erythritol molecules before and after hydrolysis showed that the free terminal carbon atom in the disaccharide had the same C^{14} content as C-1 in the free D-erythritol molecule (Table II). Thus 4-*O*-β-D-manno-

pyranosyl-D-erythritol (structure II) is the structure of the disaccharide molecule.

When D-glucose-6-C^{14} was used as a precursor in the synthesis of the disaccharide, the mannose portion was predominantly labelled in the 6-position with little randomization, indicating direct transformation of one moiety to the other (Table I). D-Erythrose-4-C^{14} and D-erythritol-4-C^{14} were very

TABLE I

Relative C^{14} incorporation into products

	Precursor		
	D-Glucose-6-C^{14}	D-Erythrose-4-C^{14}	D-Erythritol-4-C^{14}
Product	Total C^{14} in product/total C^{14} in glucose medium		
Mannopyranosyl erythritol	1.25	1.93	4.72
D-Mannose from mannopyranosyl erythritol	0.74	0.16	0.16
Erythritol from mannopyranosyl erythritol	0.59	1.81	4.47
Free D-mannitol	1.02	2.09	—
Free erythritol	—	1.80	—

TABLE II

Distribution of C^{14} in products

		Precursor		
		D-Glucose-6-C^{14}	D-Erythrose-4-C^{14}	D-Erythritol-4-C^{14}
Product	Portion of product	C^{14} as % of total C^{14} in compound		
Mannopyranosyl erythritol	C-1 of D-erythritol	3	3	5
D-Mannose from mannopyranosyl erythritol	C-1*	80	—	—
	C-6	7	—	—
D-Erythritol from mannopyranosyl erythritol	C-1	5	0	1
	C-2+C-3*	0	2	0
	C-4*	96	97	100
Free D-erythritol	C-4*	—	100	—
Free D-mannitol	C-1+C-6	74	76	—

*The specific activities of these carbon atoms are obtained by difference.

inefficient, however, in forming labelled mannose, but these two compounds furnished, as did D-glucose-6-C^{14}, the disaccharide with D-erythritol labelled almost exclusively in C-4. It appears that the fungus is able to reduce D-erythrose-4-C^{14} to D-erythritol-4-C^{14}, probably by a dehydrogenase, and then enables mannose to combine with C-4 of the D-erythritol molecule by a specific transglycosidation reaction.

As can be seen from the data in Table I, the amount of C^{14} contained in the erythritol molecule is less than that of the D-mannose or the D-glucose-6-C^{14} that was used as precursor in this instance. Thus, although the activity is located in C-4 of the D-erythritol molecule, its diminished value indicates that the compound is not formed simply by C-2—C-3 bond scission of the glucose, but that randomization has taken place in its formation. This dilution effect has been shown to exist in the formation of D-erythritol-4-C^{14} from D-glucose-6-C^{14} using the osmophilic yeast *Torulopsis magnoliae*. In this case the mechanism of erythritol biosynthesis has been shown to be complex (9).

When an attempt was made to synthesize disaccharides by adding to the medium other alcohols such as D-mannitol, ribitol, glycerol, or ethanol a copious production of erythritol was observed. Thus, either the ability of the organism to form the appropriate mannose derivative used in hexose transfer is affected, or the enzyme necessary for the transglycosidation reaction is inhibited by these alcohols.

In the radioactive experiments using D-glucose-6-C^{14} and D-erythrose-4-C^{14} as precursors, small amounts of D-mannitol were isolated. Unexpectedly, with the D-erythrose-4-C^{14} precursor the activity of the D-mannitol was exceptionally high, far greater than that of the D-mannose portion of the disaccharide. This effect was noted, too, in the instance when L-erythrulose-1-C^{14} was used in the medium. It is evident that the formation of mannose and mannitol are not closely related and that perhaps the four-carbon fragments are incorporated into mannitol via fructose which is formed through the pentose phosphate shunt (14). It is hoped that the metabolism of L-erythrulose by the organism will be discussed in a forthcoming publication.

Acknowledgments

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EFFECTS OF EXPOSURE TO COLD ON ACETYLATION IN THE RAT[1]

JAMES CAMPBELL, GORDON R. GREEN, AND HARVEY SOCOL

Abstract

Acetylation of *p*-aminobenzoic acid in rats was decreased by exposure to cold. The result suggests that in these animals competition for active acetate by heat-yielding processes may limit its availability for acetylation.

Introduction

The ability to acetylate arylamines, including *p*-aminobenzoic acid (PABA) and sulphonamides, is possessed in varying degree by different mammals (1). The chief site of acetylation in the body is thought to be the liver (2, 3, 4), although it has been suggested that the process may also occur in other organs (5). Lipmann demonstrated in 1945 that acetylation could be effected enzymatically by a system extractable from tissues, particularly pigeon liver, and that a heat-stable factor (the cofactor of acetylation or CoA) was necessary for the reaction (6). A constituent of this cofactor is pantothenic acid (7). The investigations of Lynen and his colleagues revealed that "active acetate" produced by respiring yeast was acetyl CoA (8). These discoveries had a remarkable effect on research; in a brief period of time it was shown that acetyl CoA was involved in many enzymatic reactions and was apparently the long-sought-for "active two-carbon moeity" of fatty acid metabolism in the body (8, 9).

Riggs and Hegsted (10) found that feeding a pantothenate-deficient diet reduced acetylation in the rat, presumably by decrease in the CoA available for this activity. Studies on CoA in tissues showed that exposure of rats to cold increased the concentration and amount of this substance extractable from the liver (11). These facts led to the present investigation of the acetylating activity of the rat exposed to cold.

Methods and Experimental Procedures

Adult, male Wistar rats were kept in individual cages at the environmental air temperatures of either 22–23° C (room temperature) or 0–2° C (cold temperature). The four groups of rats were fed a complete diet (No. CB-3) for 13 days prior to the experiment. Two groups were then transferred to the cold room. One group continued to receive the complete diet while the other was fed a diet deficient in pantothenate (No. CB-4); the compositions of these purified diets have been detailed (11). The other two groups remained at room temperature, and one of them received the pantothenate-deficient diet.

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For the test of acetylation, each rat received 2.5 mg of PABA in saline intraperitoneally, and the urine was collected in the following 24 hours. Each rat was fed 22 g of brown sugar during this period, instead of the usual diet, in order to reduce contamination of the urine with the food and yet to maintain the caloric intake in the cold. The urine together with the washings from each cage was made up to 100 ml and filtered. The free and the total PABA were determined by the method of Bratton and Marshall (12), and the bound, assumed to be acetylated, was calculated by difference. The probabilities of differences between means were calculated according to Fisher (13).

Results

The recoveries of total PABA in the urine from the rats at cold and room temperatures were 66 and 52% of the dose injected respectively. The percentage of acetylation of PABA (percentage of the total urinary PABA in the bound form) was less in the rats exposed to the cold environment than in those at room temperature. Statistical analysis of the results showed that the difference, due to these environments, was significant both for the groups fed the complete diet, and for those fed the pantothenate-deficient diet. The difference between all rats in the cold and all rats at room temperature was highly significant (Table I).

TABLE I

Acetylation by rats

Treatment			No. of rats	PABA excreted, mg per rat, means			Bound PABA % of total in urine, means ± S.E.
Environmental air temperature, °C	Duration, days	Diet type		Total	Free	Bound	
1	3	Complete	10	1.61	1.42	0.19	12.4±0.98
1	3	Pantothenate-deficient	10	1.77	1.52	0.25	14.2±1.27
						Mean	13.3±0.81
23	3	Complete	9	1.24	0.88	0.36	29.0±4.77
23	3	Pantothenate-deficient	8	1.33	1.05	0.28	22.5±3.68
						Mean	26.0±3.07
Probabilities of differences between means:							
Complete diet: 1° C vs. 23° C							<0.005
Pantothenate-deficient diet: 1° C vs. 23° C							<0.05
All rats at 1° C vs. all rats at 23° C							<0.001

NOTE: Male rats of 240 to 350 g body weight were each given 2.5 mg of *p*-aminobenzoic acid (PABA) intraperitoneally, and the urine was collected during the next 24 hours.

The alterations of diet had no apparent influence on the degree of acetylation in these animals. This cannot be considered to disagree with the results of Riggs and Hegsted (10) because in our experiments adult rats were given the deficient diet for only 3 days.

Discussion

The feeding of diets deficient in pantothenate decreases both the acetylating activity of the rat (10) and the CoA extractable from the livers of these animals (11, 14, 15). Exposure of the rat to cold raises the CoA content of the liver (11). It was therefore contrary to our expectations to find reduced acetylating

activity under these conditions. This observation in experimental animals may be related to the findings of Blondheim and his colleagues that in man during cold weather the blood has lower acetylating activity in vitro than during warm weather (16).

Since many investigations indicate that active acetate is required for acetylation in the body, an explanation of the present results is offered on the basis of the formation and utilization of active acetate, as illustrated in Fig. 1.

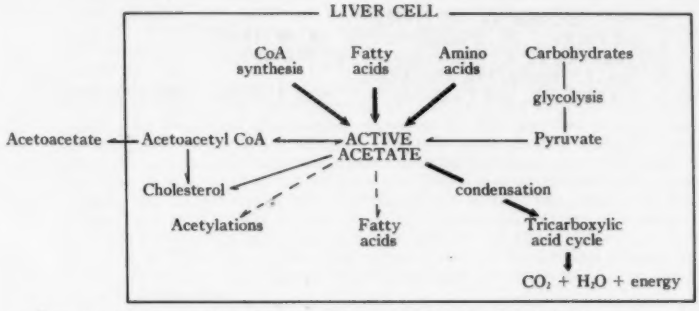

FIG. 1. Known and postulated changes in the rates of metabolic processes in liver tissue of animals exposed to cold. The signs ⟶ and - - - - → indicate increased and decreased rates, respectively, as compared with animals at room temperature. The references are given in the text.

In laboratory animals exposed to cold, as compared with those under usual conditions, there is increased utilization of energy (17), with decrease in body weight and in reserves of depot fat (11). The liver has decreased stores of glycogen (18) and increased oxidative activity (19, 20). Consequently increased formation of active acetate derived from fatty acids, amino acids, and carbodydrates would be expected to occur in the livers of these animals. The increased amounts of CoA are presumably available for the formation of active acetate. On the other hand, the utilization of active acetate for acetylation in the body and for the synthesis of fatty acids in the liver is reduced (21). More of it should therefore be available for utilization by alternative paths. One of these is the condensation reaction, whereby active acetate reacts with oxalacetate to form citrate (9). It seems likely that when rats are exposed to cold a higher proportion of the available active acetate may be channeled into the tricarboxylic acid cycle. This would be consistent with the demands of these animals for heat production, their decreased body size, and small energy reserves.

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EFFECTS OF EXPOSURE TO COLD AND OF DIET ON COENZYME A LEVELS IN TISSUES[1]

JAMES CAMPBELL, GORDON R. GREEN, EDUARD SCHÖNBAUM, AND HARVEY SOCOL

Abstract

Exposure of adult rats to a cold environment increased the coenzyme A (CoA) extractable from their livers. The increases, in rats fed a complete diet, were found within 3 days, and were larger the longer the duration of exposure, amounting to 6, 27, and 60% above the control values after 3, 17, and 24 days respectively. The relations were similar when the CoA from the liver was expressed per unit weight of fresh tissue, or of liver total solids, or of whole body. When rats were fed a diet deficient in pantothenic acid, exposure to cold also resulted in elevation of CoA in the liver, but the differences did not progress with the duration of exposure. The liver CoA was further increased by the addition of large amounts of ascorbic acid to both the complete and the pantothenate-deficient diets. This effect of ascorbic acid occurred in rats under both ordinary and cold environmental conditions. Exposure to cold did not alter the concentration of CoA in the adrenal glands, but caused enlargement of the glands so that the amount of CoA in the adrenal per unit weight of the whole body increased. Other alterations in these animals exposed to cold included enlargement of the kidneys and heart, and decrease in depot fat. Deficiency of pantothenate in the diet slowed the rate of growth of young rats and significantly reduced the levels of CoA in the liver and the adrenal glands.

Introduction

Coenzyme A (CoA) is necessary for many enzymatic reactions involving fatty acids, amino acids, carbohydrates, and metabolic products from them (1). It is apparently the physiologically active form of pantothenic acid and occurs in relatively high concentrations in the liver and the adrenal glands (2, 3). Dietary deficiency of pantothenate results in reduced CoA levels in tissues, with characteristic signs of deficiency (4, 5). On the basis of a general postulate that changes in metabolic states should induce alterations in the amounts of cofactors in tissues, we have investigated the influences of exposure to cold and of dietary factors on CoA levels. It is obvious that the turnover of the cofactor is of first importance, but measurement of it is at present beyond our technical resources. Experiments, reported briefly, showed that the CoA content of liver tissue was increased by exposure of rats to a cold environment (6). This has been confirmed in adult rats by Brumleve and Olson (7). The present studies extend these results and demonstrate conclusively that exposure to cold increases the amount and concentration of CoA in the liver.

Methods, Materials, and Experimental Procedures

Male rats of the Wistar strain were kept in individual cages with wire mesh bottoms at the usual environmental temperature af 22 to 23° C (room temperature) or in the cold at 0 to 2° C in rooms with circulation of air. The

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purified isocaloric diets all contained 21% of protein, 12% of fat, and 60.5% of carbohydrate (Tables I and II). The protein of diets Nos. CB-1 to -4 was

TABLE I

Compositions of the diets

Components	Diets, numbers and compositions, g per 100 g of diet							
	CA-1	CA-2	CA-3	CA-4	CB-1	CB-2	CB-3	CB-4
Casein	6	6			21	21	21	21
Peanut meal, extracted*	12	12						
Soya protein †	9	9						
Corn oil	4	4			4	4	4	4
Shortening (Primex)	7	7			7	7	7	7
Sucrose	34.5	34.5			40.5	40.5	40.5	40.5
Dextrin	10	10			10	10	10	10
Corn starch	10	10			10	10	10	10
Cellu flour	2	2			2	2	2	2
Salt mix (8)	3	3			3	3	3	3
Choline chloride	0.5	0.5			0.5	0.5	0.5	0.5
Vitamins A, D, E in oil (8)	1	1			1	1	1	1
Vitamin mix, No. PDX	1				1			
Vitamin mix, No. PDT		1				1		
Vitamin mix, No. PDA							1	
Vitamin mix, No. PDB								1
Diets with ascorbic acid, numbers and compositions								
Diet No. CA-1			98					
Diet No. CA-2				98				
Ascorbic acid			2	2				

NOTE: The diets were complete (Nos. CA-1; CA-3; CB-1; and CB-3) or deficient in pantothenate (Nos. CA-2; CA-4; CB-2; and CB-4). Diets Nos. CA-3 and CA-4 contained ascorbic acid.
*Solvent-process peanut meal, further extracted with hot alcohol (8).
†Drackett Assay Protein (Cl). The Archer–Daniels–Midland Co., Cincinnati.

TABLE II

Vitamin mixes

Components	Numbers and compositions of mixes, g per kg of mix			
	PDX	PDT	PDA	PDB
Biotin	0.03	0.03	0.03	0.03
Thiamin HCl	0.05	0.05	0.05	0.05
Riboflavin	0.25	0.25	0.25	0.25
Pyridoxine HCl	0.20	0.20	0.20	0.20
Nicotinic acid	1.00	1.00	1.00	1.00
Folic acid	0.05	0.05	0.05	0.05
Menadione	0.10	0.10	0.10	0.10
Inositol	50.00	50.00	50.00	50.00
Calcium pantothenate	1.00		1.00	
p-Aminobenzoic acid	10.00	10.00		
Vitamin B_{12} triturate*	3.00	3.00		
Sucrose, 40-mesh	933.87	934.87	946.87	947.87

*Vitamin B_{12} triturated with mannitol, 1 g mix = 1 mg B_{12}.

supplied by casein, and of diets CA-1 to -4 by casein, fat-free peanut meal further extracted with alcohol (8), and soya protein. These diets differed in the addition or omission of pantothenic acid or of ascorbic acid. Minor differences were that vitamin B_{12} and *p*-aminobenzoic acid were not included in

diets CB-3 and CB-4. The purified complete diets were fed ad libitum for 7 to 10 days prior to exposure, and the dietary alterations were made at the beginning of exposure. Food consumption was measured daily (amount provided less amount left) and the body weights were recorded on alternate days.

The rats were killed by decapitation and samples of tissue were dissected, weighed, and frozen within 3 minutes. CoA was extracted by homogenizing approximately 100 mg of tissue in a Potter–Elvehjem tube with 19 volumes of water, rapidly heating to boiling, and separating the fluid. The amount of CoA thus extracted from the tissue, in Lipmann units, was determined in early experiments by the method of Kaplan and Lipmann (9). Later, the enzymatic acetylation of *p*-nitroaniline by pigeon liver enzyme was used for the determination of CoA (10). Each tube contained sodium citrate (19 μmoles); sodium acetate (24 μmoles); adenosine triphosphate (4 μmoles); *p*-nitroaniline (0.4 μmole); tris(hydroxymethyl)aminomethane buffer, pH 7.6 (100 μmoles); cysteine hydrochloride (15 μmoles); appropriate amounts of tissue extract, or of water, or of standard solution of CoA; and finally 0.2 ml of the enzyme solution. The final volume was 1.1 ml. In assays of about 15 tissue samples at a time were included two tubes with different amounts of each tissue extract, three tubes with different amounts of standard solution of CoA (in place of the extract), two tubes without enzyme and tissue extract (blanks), and two tubes without tissue extract but with enzyme (for residual CoA in the enzyme solution). The enzyme was obtained by extraction of pigeon liver acetone powder (9), and CoA was removed from it by trituration with Dowex 1-X2 resin, 200–400 mesh (11). After incubation at 37° C for 1 hour the proteins were precipitated by the addition of 0.2 ml each of solutions of zinc sulphate and sodium hydroxide and the optical density of the filtrate was measured at 420 mμ. The CoA of the liver of each rat was determined as above; the adrenal glands from each group of rats were combined for analysis. Samples of the liver were dried at 110° C to constant weight to give the total solids. The standard errors (S.E.) and the probabilities of differences between means (12) are given in the tables.

Results

Effects of Exposure to Cold

In preliminary experiments, the CoA extractable from the livers of mature rats exposed to cold for 1 day was 21% above that of controls at room temperature, but the difference was not statistically significant. After 3 days of exposure the CoA extractable from the liver was twice that of the control value and the difference was significant (Table III). Since the methods employed for determination of CoA differed in experiments 1 and 2, these comparisons are valid within, but not between, these particular experiments. In all subsequent work the later method (10) was used.

Further experiments, with variations in diet and in duration of exposure, were carried out. Although various diets were fed, the levels of CoA in the liver were higher in all the groups of rats exposed to cold than in the corresponding groups at room temperature (Table IV). This relation held for

TABLE III
Effects of exposure to cold on liver and adrenal CoA

No. of expt. and ref. to method	Environmental temp. and duration	No. of rats	CoA units, means ± S.E., extractable from Liver: Per g fresh tissue	Liver: Per g total solids	Liver: Per 100 g body wt.	Adrenal: Per g fresh tissue	Adrenal: Per 100 g body wt.
1. (10)	23° C, 1 day	6	133 ± 13.3	377 ± 31.7	499 ± 47.3	43	0.58
	1° C, 1 day	6	161 ± 9.5	475 ± 30.2	657 ± 42.8	41	0.76
2. (9)	23° C, 3 days	14	77 ± 6.9		324 ± 35.3		
	1° C, 3 days	10	154 ± 12.9		583 ± 70.2		
Probabilities of differences between means							
Groups							
23° C vs. 1° C, 1 day			<0.2	<0.05	<0.05		
23° C vs. 1° C, 3 days			<0.001		<0.001		

NOTE: Male rats of 165 to 281 g body weight were fed chow and kept at the environmental temperatures indicated.

CoA calculated per gram of fresh tissue, or per 100 g of body weight. The differences were statistically significant in 10 of the 11 pairs of groups on the fresh tissue basis of calculation, and in 9 of the 11 pairs on the body weight basis. The relations were the same also when CoA was calculated per gram of total solids of the liver; these differences were significant in 10 of the 11 pairs of groups (Fig. 1). The increases in the content of CoA in the liver became larger the longer the duration of exposure to cold, in rats fed the complete diet (Fig. 1). The influences of diet on this response are described below.

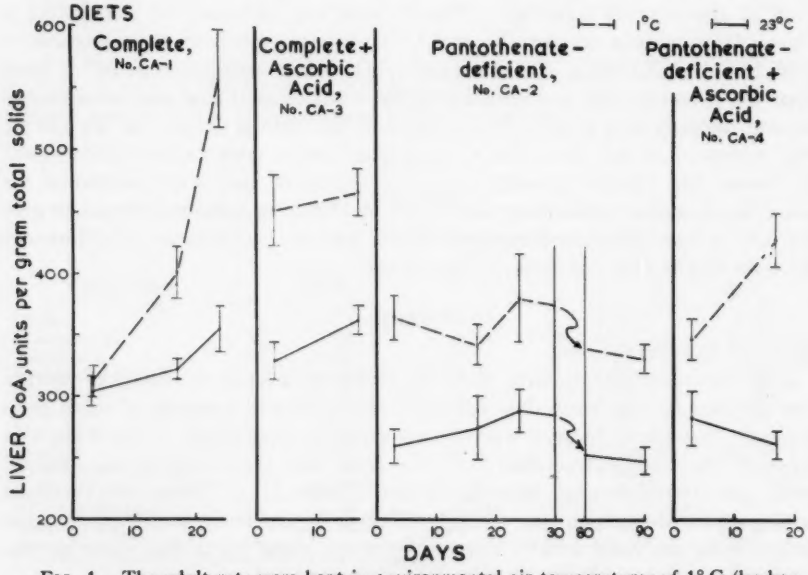

FIG. 1. The adult rats were kept in environmental air temperatures of 1° C (broken lines) or of 23° C (continuous lines). The standard errors of the mean CoA extractable from the liver are shown by the vertical lines.

The CoA extractable from the adrenal glands of the groups of rats described in Table IV ranged from 25 to 34 units per g of fresh tissue. This is about

TABLE IV

Effects of diet and of exposure to cold on liver CoA

Environmental conditions	Diet Nos.	Extractable coenzyme A of the liver, units, means ± S.E.							
		3 days		17 days		24 days		90 days	
		Per g f.t.	Per 100 g b.w.	Per g f.t.	Per 100 g b.w.	Per g f.t.	Per 100 g b.w.	Per g f.t.	Per 100 g b.w.
R.T.	CA-1, CB-3	(6) 104±4.3	453± 9.4	(6) 103±3.5	431±15.6	(9) 103± 5.0	397±23.8		
C.T.	CA-1, CB-3	(6) 110±5.2	472±33.8	(5) 131±6.8	617±22.0	(8) 165±11.0	672±54.2		
R.T.	CA-2, CB-4	(6) 87±4.0	384±21.5	(6) 91±5.4	392±23.2	(10) 80± 4.8	290±16.7	(5) 76±2.8	261±14.8
C.T.	CA-2, CB-4	(6) 123±6.5	537±38.5	(10) 117±5.2	503±25.5	(10) 111±11.0	439±47.9	(7) 97±3.5	389± 9.0
R.T.	CA-3	(6) 112±5.8	486±38.8	(6) 117±2.5	497±20.9				
C.T.	CA-3	(6) 153±9.3	669±57.4	(6) 153±6.0	653±41.8				
R.T.	CA-4	(6) 98±7.5	438±41.2	(6) 94±4.4	407±18.4				
C.T.	CA-4	(6) 120±6.4	516±31.6	(6) 144±6.8	629±25.9				
Probabilities* of differences between means									
R.T. vs. C.T.									
With diets CA-1, CB-3		N.S.	N.S.	<0.005	<0.001	<0.001	<0.001		
With diets CA-2, CB-4		<0.001	<0.01	<0.01	<0.01	<0.025	<0.01	<0.005	<0.001
With diet CA-3		<0.005	<0.05	<0.001	<0.01				
With diet CA-4		<0.05	N.S.	<0.001	<0.001				
Diets at R.T.									
CA-1, CB-3 vs. CA-2, CB-4		<0.025	<0.025	<0.1	N.S.	<0.005	<0.005		
CA-1 vs. CA-3		N.S.	N.S.	<0.01	<0.05				
CA-2 vs. CA-4		N.S.	N.S.	N.S.	N.S.				
Diets at C.T.									
CA-1, CB-3 vs. CA-2, CB-4		N.S.	N.S.	N.S.	<0.025	<0.01	<0.01		
CA-1 vs. CA-3		<0.005	<0.025	<0.05	N.S.				
CA-2 vs. CA-4		N.S.	N.S.	<0.01	<0.01				

Male rats of 190 to 230 g body weight were kept for various periods of time at the ambient temperature of 22–23° C (R.T.), or of 0–2° C (C.T.) and were fed diets that were complete (Nos. CA-1 and CB-3); or deficient in pantothenate (Nos. CA-2 and CB-4); or complete with ascorbic acid added (No. CA-3); or pantothenate-deficient with ascorbic acid added (No. CA-4). The coenzyme A extractable from the liver is given in units per g of fresh tissue (f.t.) or per 100 g of body weight (b.w.). The numbers of animals per group are given in parentheses.

*Probabilities of 0.1 or above are indicated as N.S. (not significant).

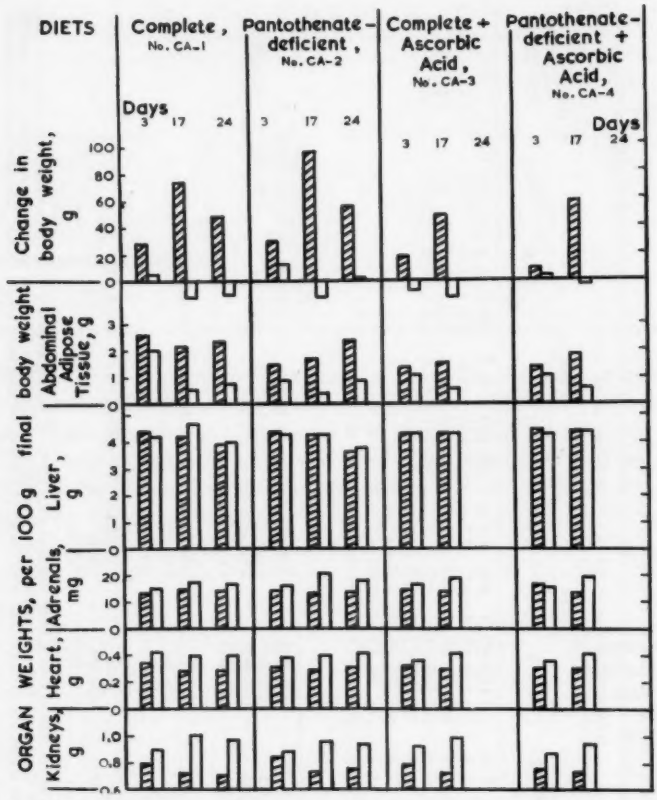

FIG. 2. The adult rats were kept at the environmental temperatures of 23° C (cross-hatched columns) or of 1° C (open columns).

one-fourth the concentration of CoA in liver tissue. Exposure to cold did not alter significantly the concentration of CoA in the rat adrenal glands, but caused enlargement of the glands (Fig. 2), with one exception (diet CA-4, 3 days). In 7 of the 11 pairs of groups the amount of CoA in the adrenals per 100 g of rat was higher by 17 to 60% than in the corresponding groups at room temperature. At the 3-day interval, however, there was no difference in two pairs of groups and in a third (diet deficient in pantothenate, with added ascorbic acid) there was an inversion of this difference. Comparing all groups of rats in the cold with those at room temperature, the probability of the increase in adrenal CoA on a body weight basis was 0.005–0.01.

The rats kept in the cold either lost weight, or gained weight relatively slowly, and their reserves of depot fat, represented by the weights of the abdominal adipose tissues, were always less than in those at room temperature (Fig. 2). These reductions in body weight and stored fat occurred despite increased intakes of food (Table V). Exposure of the rats to cold caused

enlargement of the adrenal glands, heart, and kidneys relative to body weight. The liver weight was not altered, however, except for 1 of 10 comparisons, i.e. complete diet for 17 days (Fig. 2).

TABLE V
Food intakes

Environmental temperature, °C	Food intakes, daily means ±S.E., g per rat			
	3 days	17 days	24 days	90 days
23	19.3±0.31	18.7±0.37	19.6±0.20	16.0±0.24
1	22.5±1.01	21.1±0.27	22.0±0.33	21.9±0.45

NOTE: The food intakes were determined daily. The mean values for the rats of Table IV are given at the intervals selected.

Effects of Pantothenic and Ascorbic Acids in the Diet of Adult Rats

Groups of adult rats were fed either purified diets deficient in pantothenic acid or corresponding complete diets. At room temperature, the liver CoA was significantly less in the rats fed the deficient diet for 3, 17, and 24 days (Table IV). In the cold environment, for 17 and 24 days, the liver CoA was also less in the rats fed the pantothenate-deficient diet, but the differences were not as definite. When rats were fed the pantothenate-deficient diets, the liver CoA in the groups exposed to cold was significantly higher than in the groups at room temperature. This was observed after 3 days of exposure, and continued up to 90 days. However, this change was not progressive with time, i.e. the curve for the pantothenate-deficient rats in the cold roughly paralleled that of their controls at room temperature (Fig. 1).

Higher concentrations and amounts of CoA were found in the livers of the rats that were given large amounts of ascorbic acid in the diet. This result occurred in rats both at room temperature and in the cold. It was obtained in seven of the eight pairs of groups, and the difference was statistically significant in four of these pairs (Table IV).

The addition of ascorbic acid to the complete diet resulted in statistically significant reductions in body weight gain in rats at room temperature for 3 and 17 days, and in rats in the cold for 3 (but not for 17) days. It also reduced significantly the amount of adipose tissue present in rats both at room temperature and in the cold for 3 days. At 17 days, however, the difference was not significant. The addition of ascorbic acid to the pantothenate-deficient diet significantly reduced body weight gain in rats at room temperature for 3 and 17 days.

Effects of Pantothenate-deficient Diets on Growth and on the CoA of Tissues

The diets whose effects are described above were tested for growth-promoting activity and were compared to similar diets that contained casein as the only source of protein. Weanling female rats of uniform mean initial body weight (40 g) were kept at ordinary environmental temperature. The food was supplied ad libitum and the intake was determined daily. The two complete diets produced almost the same rates of body weight gain, although that containing extracted peanut meal, soya protein, and casein was slightly more

effective than that with casein alone as the source of protein (Fig. 3). However, these diets were the same in respect of the amount of food required to produce a unit gain in body weight, and of the amount of abdominal adipose tissue present at the end of the experiment (Table VI).

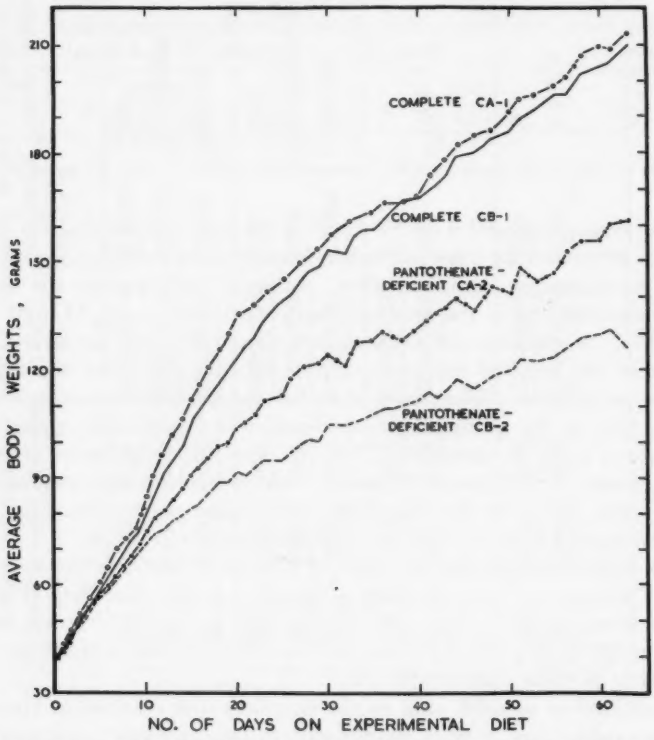

FIG. 3. The growth curves of weanling female rats at room temperature, fed different experimental diets.

The rats fed the pantothenate-deficient diets grew at reduced rates (Fig. 3); their food intake/body weight gain ratios were above normal and their stores of abdominal fat tissue were low (Table VI). They appeared to be excitable and scattered much of the food presented to them. Thinning of the fur and staining with porphyrin around the eyes, nostrils, forehead, and paws were also observed. (The individual growth curves of these animals were somewhat erratic, but plateaued after 30 days in all but one instance. This exceptional animal continued to grow at an almost normal rate.) The pantothenate-deficient diet with casein as the sole source of protein (No. CB-2) gave the poorest growth, the highest food intake/body weight gain ratio, and the least amount of adipose tissue at the end of the experiment. No deaths occurred under these dietary conditions.

TABLE VI

Growth of rats fed pantothenate-deficient diets

Diet		No. of rats	Body weight, means		Food intake		Ratio, food intake/ body dry wt. gain, g/g	Adipose tissue, abdominal per 100 g of body weight, g
Type	No.		Final. g	Change from initial, g	Per rat per day,* g	Total in 63 days, g		
Complete	CB-1	6	210± 6.8	169± 7.8	9.4±0.28	592	14.1	6.59±0.71
Pantothenate-deficient	CB-2	6	126±13.1	89±12.6	9.0±0.24	567	25.8	1.91±0.73
Complete	CA-1	5	213± 6.5	174± 9.3	9.9±0.30	624	14.2	6.25±0.52
Pantothenate-deficient	CA-2	6	161±12.7	122±11.1	9.6±0.31	605	19.5	3.54±0.69
Probabilities of differences between means								
CB-1 vs. CB-2			<0.001	<0.001	N.S.†			<0.005
CA-1 vs. CA-2			<0.01	<0.01	N.S.†			<0.025

NOTE: Weanling female rats, mean initial body weight of 40 g per group, were fed the purified diets, either complete or deficient in pantothenate, during 63 days at the environmental temperature of 23° C. The gain in dry body weight was calculated on the assumption that the total body solids of the rats in all groups were 25% of the body weight.

*Standard errors of the food intakes were calculated on the variation of the group value from day to day.

†Not significant.

At the end of the feeding period (63 days) the amounts of CoA extractable from the liver and adrenal tissues of the rats given the pantothenate-deficient diet No. CB-2 were significantly less than those of the corresponding control rats (Table VII). This confirms the observations of Olson and Kaplan (4)

TABLE VII

Effect of diet on the tissue CoA of young rats

Diets		No. of rats	CoA units, means ±S.E., extracted from:			
			Liver		Adrenal	
Type	No.		Per g fresh tissue	Per 100 g body wt.	Per g fresh tissue	Per 100 g body wt.
Complete	CB-1	6	93±2.7	338± 8.7	32±2.9	0.68±0.06
Pantothenate-deficient	CB-2	6	62±4.6	201±11.4	15±0.71	0.33±0.01
Complete	CA-1	5	76±7.2	280±28.3	25±1.0	0.59±0.02
Pantothenate-deficient	CA-2	6	69±3.4	238±16.2	24±2.0	0.61±0.05
Probabilities of differences between means						
CB-1 vs. CB-2			<0.001	<0.001	<0.025	<0.025
CA-1 vs. CA-2			N.S.*	N.S.	N.S.	N.S.

NOTE: The amounts of CoA extractable from the liver and the adrenal glands of the young female rats described in Table VI were determined.

*Probability of 0.1 or above.

and of Klein and Lipmann (5). In the rats fed the other pantothenate-deficient diet (No. CA-2) the mean CoA of the liver was also less than in the controls, but the difference was not significant, and the adrenal CoA of these groups was the same.

Discussion

In the experiments described, exposure of rats to cold resulted in increased CoA levels in the liver. In view of the increased metabolic activity resulting from exposure to cold, it is unlikely that this effect would be caused by de-

creased loss of CoA from the cells. It is more probable that the effect may be due to increased rate of synthesis. However, this response also occurred when the animals were fed a purified diet deficient in pantothenate. The explanation for this is unknown, but the possibilities include (*a*) the derivation of precursors of CoA from endogenous sources; (*b*) the absorption of pantothenate derived from the intestinal flora, or by coprophagy (14); and (*c*) the synthesis of pantothenate in limited amounts by animal tissues. Although Causi and Romano (15) found that ascorbic acid caused a decrease in the CoA of the liver in rats within 5 days, in our experiments CoA levels were either unchanged or increased by feeding ascorbic acid. The physiological significance of this is not clear, but we wish to note the results of two other investigations that may be related. Daft and his colleagues (16) found that weanling rats could be raised to normal adult weight when fed diets deficient in pantothenic acid, if ascorbic acid was provided. Dugal and his colleagues (17) obtained evidence that ascorbic acid favored survival of animals exposed to cold.

Tabachnick and Bonnycastle (18) found that the production of a hyperthyroid state in rats increases the CoA of the liver. Other results indicate that exposure to cold induces increased thyroid activity (19, 20, 21). Hyperthyroidism and low environmental temperature both produce certain other common changes including increased metabolic rate (22, 23), decreased body weight and depot fat reserves, increased adrenal gland weight (24), and increased oxidative activity of liver tissue (25). Diminished acetylating activity is found in hyperthyroidism in man (26) and in the rat (27), and occurs also in the rat exposed to cold (28).

This comparison suggests that the increase in tissue CoA in response to cold may be dependent on increased thyroid activity. Another possibility to be considered is that the increase in the CoA of the liver may be a consequence of a common metabolic state induced in the tissues of animals under both these conditions. This state in the tissues may be described as including increased oxidative activity and utilization of energy, with increased heat production and competition for available substrates for heat-yielding processes. Further experiments will be necessary to provide an explanation.

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